volume 23, no. 4

july-aug. 1958

BIOCHEMISTRY

БИОХИМИЯ

biokhimiia

translated from Russian

CONSULTANTS BUREAU, INC.

ANNOUNCING

a new journal in the field of
Biological Sciences
—translated from Russian

Antibiotics

(Antibiotiki)

Translated and published with the assistance of
the following companies in underwriting a
portion of the expenses involved:

ELI LILLY AND CO., INC.
THE UPJOHN COMPANY
BRISTOL LABORATORIES, INC.
CHAS. PFIZER AND CO., INC.

Published Bimonthly
Starting with No. 1, 1959

ANNUAL SUBSCRIPTION: $25.00

CONSULTANTS BUREAU, INC.
227 WEST 17TH STREET, NEW YORK 11, N. Y

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BIOKHIMIIA

A publication of the Academy of Sciences of the USSR

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CONSULTANTS BUREAU, INC.

227 W. 17th St., NEW YORK 11, N. Y.

Year and issue of first translation:
volume 21, no. 1, jan.-feb. 1956

Annual Subscription	*$20.00*
Single Issue	*10.00*

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UTILIZATION OF HIPPURIC ACID BY CERTAIN MOLDS

F. T. Sukhenko

Chair of Biochemistry, Novosibirsk Medical Institute

Peptone, plant and animal proteins, amino acids, and urea can serve as nitrogenous nutrients for the growth of molds, including, according to our findings [1], Fusaria and Microspora. We found that ammonia was always present in the fluid media in which these molds had been grown, when they contained the above nutrients. The impression was given that the carbon – nitrogen bond had to be split before these molds could utilize the nutrients.

A peculiarity of hippuric acid is that its carbonyl carbon enters into a peptide bond joined to a benzene ring, and this may so modify the peptide bond as to protect it from the action of the enzymes of some molds. That certain molecular groupings are capable of protecting the carbon – nitrogen bond emerges from the work of Brunel [1a] on the utilization of urea and allantoin by Sterigmatocystis nigra.

Consequently, we decided to clarify whether or not Microspora, Fusaria, and other fungi can use hippuric acid for nutrition and to what extent their growth can be assured by this single source of nitrogen.

MATERIALS AND METHODS

We carried out two successive series of experiments. We used the following molds: Microsporum lanosum (Strain 2), Microsporum ferrugineum (Strains K and KK), a Fusarium of the Sporotrichiella group (Strain 1045). In addition to these, we used Aspergillus niger and Trichoderma lignorum in the second series of experiments. The molds were cultured in a nutrient medium containing about 3% of glucose, 0.4% of hippuric acid, mineral salts ($MgSO_4 \cdot 7H_2O$ – 0.025%, NaCl – 0.025%, KH_2PO_4 – 0.05%, $FeSO_4$ – 0.025%), with small amounts of vitamins B_1, B_2, and PP. The media were sterilized by passage through a Seitz filter, and 95 ml portions were placed in 250 ml conical flasks in the first series of experiments, and, in addition, 45 ml portions in 100 ml flasks in the second series. Each species of mold was inoculated into 20 flasks in the first series, and into 10 flasks in the second. Suspensions of mycelia of Microspora and Fusarium, grown in a glucose-peptone medium, were used for the inoculations. The suspensions were prepared as described by us earlier [2]. The inocula of other species of molds consisted of spores. The cultures were grown under the usual laboratory conditions.

After separating the mycelia, we analyzed the medium for the following: total nitrogen, by the Kjeldahl method, amino acid nitrogen (glycine), according to Pope and Stevens, ammonia, by Conway's method, glucose by Issekutz's method, and hippuric acid, from the amount of glycine produced by its hydrolysis. Hydrolysis of hippuric acid was effected by Asatiani's procedure [3].

As a control, we tested the medium for the presence of glycine, benzoic acid, and hippuric acid, by a chromatographic method, using "Fast" chromatographic paper made by the Leningrad Factory. Benzoic and hippuric acids were extracted with ether from the nutrient medium or the reaction mixture. The extracts were evaporated to dryness. The residue was dissolved in water, and the solutions were filtered and chromatographed. We used a number of solvent systems as the mobile phase. The most convenient were found to be n-butanol – acetic acid – water (4 : 1 : 1) and n-butanol – ethanol – 1.5 N ammonium carbonate (2 : 1 : 1), with addition of ammonia. We applied ascending chromatography at room temperature, with a length of run of 20 – 24 hr ; spraying was effected according to Friedler and Smith [6], with the difference that the iodide concentration taken was a third to a half of that recommended by these authors. Ninhydrin was used as the spray reagent for glycine. The substances were identified by comparison with reference spots.

EXPERIMENTAL RESULTS

1. Short Description of Growth of the Molds

The first visible signs of growth of Microspora in hippuric acid media appeared on the 5th – 6th day of incubation. Thereafter the mold grew very slowly. During the first period of growth Microsporum lanosum formed numerous small discrete colonies, attached to the bottom of the flasks. The colonies then became confluent, and mycelial buds began to grow up towards the surface of the medium. Within 6 weeks, or sometimes even longer, mycelia appeared at the surface of some of the flasks, later forming a very thin, cobweb-like, whitish film on the surface. The downy aerial mycelium typical of this species did not form.

Both strains of Microsporum ferrugineum also displayed bottom growth only for a long time, forming thin cobweb-like films. White mycelia then appeared at the surface, eventually forming a very thin whitish film. Strain K of this mold grew more slowly than did Strain KK. The initial yellow, and later rusty, coloration characteristic of this species did not appear.

The first signs of growth of Fusarium were in good evidence on the third day after inoculation. In contrast to Microsporum, the subsequent growth of this mold was fairly rapid. Thus, in the first series of experiments, the entire depth of the nutrient medium was penetrated by mycelia, and its surface was covered by a thin film, which began to assume the color characteristic of this mold. By the 32nd day a fairly thick surface film had formed, of a deep pink color.

Aspergillus spores gave white mycelia two days after inoculation into either glucose-peptone or hippuric acid media. Sporulation was inhibited by shaking the flasks from time to time for a number of days after inoculation, to ensure continued growth of the vegetative form of the mold. The rate of growth of the mold in hippuric acid medium was practically the same as in glucose-peptone medium.

The growth of Trichoderma in the hippuric acid medium was much slower than that of Fusarium or of Aspergillus, but was faster than that of Microsporum. It grew at the bottom of the flasks, and only here and there did mycelia emerge from the medium, soon after producing the dark green spores characteristic of this mold. The mold grew faster in glucose-peptone than in hippuric acid media, and, in addition to the usual dark green spores, it produced yellow spores, tinged with green, in large amounts.

It thus appears that Fusarium and Aspergillus grow abundantly in the hippuric acid medium, whereas Trichoderma and especially Microsporum grow only very slowly in this medium. This contrast in the rate of growth and of production of mycelial mass of the species of molds under consideration cannot be ascribed to the greater rate of growth of Aspergillus and Fusarium in general, as compared with Microsporum, but is due chiefly to differences in their utilization of hippuric acid.

2. Changes in the Composition of the Nutrient Media During the Process of Growth of the Molds

In view of the slow growth of Microsporum, analyses of the nutrient media were made 250 days after inoculation, in the first series of experiments, and after 54 – 58 days in the second. The results are presented in Table 1.

It is evident from the data of Table 1 that no great changes in the composition of the medium take place during growth of Microsporum. The differences found between the total and amino acid nitrogen contents of the media before and after incubation are extremely small; in some cases no difference could be found. There is little difference in the glucose content of the media. The pH of the medium is also hardly affected.

The small yield of Microsporum mycelium, and the insignificant changes in the composition of the nutrient medium, are evidence that hippuric acid is hardly at all utilized by this mold. The poor utilization of hippuric acid by Microspora, and their scanty growth in this medium, may be due either to their lack of enzymes catalyzing breakdown of hippuric acid, or to the presence of growth inhibitors in the nutrient medium. It might be supposed that hippuric acid or benzoic acid arising from its hydrolysis could exert an inhibitory effect on growth of the molds.

Growth of Trichoderma was associated with only small changes in the composition of the nutrient medium. Its pH was, however, shifted in the direction of greater acidity. This is evidence of formation of acidic products from glucose during growth of the mold.

TABLE 1

Results of Analyses of Hippuric Acid Nutrient Medium in Which Molds Have Been Grown

Species of mold	Date of inoculation of mold	Date of analysis of medium	Mycelial wt / flask, mg	pH	Glucose, mg %	Nitrogen, mg % total	Nitrogen, mg % amino acid pre-hydrolysis	Nitrogen, mg % amino acid post hydrolysis	Nitrogen, mg % ammonia
First series									
Control medium	—	19.X.1956	—	6.62	2940	31.8	none	31.8	none
Fusarium, Strain 1045	7.II.1956	30.III.1956	214	6.02	130	19.6	4.2	15.7	"
Microsporum lanosum, Strain 2	4.II.1956	16.V.1956	193	8.89	none	16.8	2.8	4.8	6.2
		9.X.1956	40	6.20	2620	30.2	none	29.6	none
Microsporum ferrugineum, Strain KK	"	"	50	6.57	2700	30.4	"	31.00	"
Microsporum ferrugineum, Strain K	7.II.1956	"	24	6.48	2740	—	"	31.3	"
Second series									
Control medium	—	14.I.1957	—	7.10	2927	33.6	none	32.2	none
Aspergillus	14.XII.1956	"	350	3.31	32	13.3	**	**	"
Fusarium, Strain 1045	8.XII.1956	12.II. 1957	*	3.66	none	3.4	—	—	traces
		4. I. 1957	147	5.22	890	21.7	2.8	18.1	none
Trichoderma	14.XII.1956	11.II. 1957	491	7.82	86	7.0	5.6	7.0	"
Microsporum lanosum, Strain 2	"	"	24	5.69	2563	30.8	2.8	—	"
		2.IV.1957	25	5.44	2433	30.1	2.0	28.0	"
		1.II.1957	36	6.71	2773	32.2	1.7	29.4	"
Microsporum ferrugineum, Strain KK	"	2.IV.1957	15	6.33	2733	32.9	traces	33.6	"
		11.II.1957	9	6.93	2873	33.6	1.4	30.8	"
		2.IV.1957	16	6.54	2701	32.9	1.1	32.9	"
Microsporum ferrugineum, Strain K	18.XII.1956	11.II.1957	10	7.12	2900	33.6	traces	32.8	"
		2.IV.1957	11	6.99	2763	33.6	"	32.9	"

* The whole of the mycelium was not taken for analysis, because of copious sporulation.

** Amino acids cannot be determined by the copper method in media in which Aspergillus has been grown.

Considerable changes in the composition of the nutrient medium were found as a result of incubation with Fusarium or Aspergillus. Thus 50 – 54 days after inoculation with Fusarium only small amounts of hippuric acid remained in the medium, and its decomposition products – benzoic acid and glycine – appeared in its place. At the same time, there was a considerable fall in the glucose content; it had all been utilized toward the end of the experiment. The yield of Fusarium amounted to 214 mg per flask in the first series, and to 490 mg in the second. All these findings show that Fusarium is able to break down hippuric acid, and to utilize the products for its metabolic requirements. The presence of glycine in the media is evidence that the rate of hydrolysis of hippuric acid by the mold exceeds that of the uptake of the products by the mold.

Changes in pH were also observed during growth of Fusarium; the medium first became more acid, and then more alkaline. Acidification took place during the period of active growth of the mold, and was associated with intensified utilization of glucose, while the subsequent rise in pH was associated with utilization of benzoic acid and of products of breakdown of glucose. Liberation of sodium previously neutralized by hippuric acid contributed to the alkalinization of the medium.

The effects of incubation with Aspergillus resembled those found with Fusarium, with the sole difference that the medium became much more acid, and remained acid throughout the experiment. We were not able to determine glycine by the copper method in media used for the growth of Aspergillus.

It may be concluded from our findings that Fusarium and Aspergillus can readily satisfy their metabolic needs by utilization of hippuric acid, but that Microsporum can do so only to a small extent, if at all. This difference must be due to differences in the biochemistry of the molds concerned. Thus it may be that Fusarium and Aspergillus differ from Microsporum in possessing an active enzyme system catalyzing the breakdown of hippuric acid, or that hippuric acid, or its product, benzoic acid, act as inhibitors on some enzyme systems of Microsporum, thus suppressing its growth, while not exerting this action on the analogous systems of the other species.

3. Presence of Hippuricase in Fusarium and in Microspora

Smorodinzew [5] has demonstrated the presence of hippuricase in various tissues of dogs, swine, horses, and cattle. Dox [6] found this enzyme in molds, and Zeller [7] in Lenzites sepiaria.

Smorodinzew came to the conclusion, on the basis of numerous experiments, that hippuricase (histozyme) is not a substrate-specific enzyme, acting only on hippuric acid. He believes that it catalyzes the hydrolysis of acylated amino acids. Otani [8] has described an enzyme which catalyzes hydrolysis of benzoyl-DL-leucyl-glycine, benzoylglycylglycine, and hippuric acid.

We have been unable to find any published reference to the presence of hippuricase in Fusaria and Microspora.

We examined Fusarium, Microsporum lanosum and Microsporum ferrugineum (Strains K and KK) for the presence of hippuricase, since deficiency of this enzyme might account for the poor growth of Microspora and Trichoderma in hippuric acid media, as compared with abundant growth of Fusarium and Aspergillus.

These molds were grown in glucose-peptone medium. Mycelium was taken from a number of flasks, washed three times with water, and divided into two portions. One portion was immediately homogenized in M/15 phosphate buffer, pH 6.9 – 7.0, to give a suspension of fresh mycelium. The other portion was extracted three times with acetone at room temperature, and then dried in vacuo. The acetone-treated preparation was then suspended in phosphate buffer, in the same way as for the fresh mycelium. The suspensions were at once taken for the experiments.

The test systems consisted of equal volumes (usually 30 – 50 ml) of suspension and of hippuric acid solution, the concentration of which amounted to about 200 mg-% in the systems. The control systems were: equal volumes of suspension and of phosphate buffer (this served as a control of benzoic acid which might have been produced by the mycelium), while the second control consisted of equal volumes of hippuric acid solution and phosphate buffer (control of spontaneous hydrolysis of hippuric acid).

The systems were incubated at 37°. Samples were taken at the beginning of the experiment, and thereafter at one or two-day intervals, and examined chromatographically for the presence of hippuric acid, benzoic acid, and glycine. The control systems were also sampled at the beginning and end of the experiments.

The chromatograms shown in Fig. 1 (a, b, and c) show that benzoic acid appears after 22 – 24 hr of incubation, and increases in amount as the experiment proceeds. Benzoic acid was not detected in any of the control systems. These results indicate that hippuric acid does not undergo hydrolysis in the absence of mycelia, but that it is hydrolyzed in the presence of fresh or acetone-dried Microsporum mycelia. Similar results were found with suspensions of Fusarium mycelium.

Suspensions of acetone-dried preparations of Microsporum lanosum and of Strain KK of Microsporum ferrugineum decompose hippuric acid at about the same rate as do suspensions of fresh mycelium. Suspensions of fresh mycelium of Microsporum ferrugineum, Strain K, decompose hippuric acid at a slightly slower rate than do suspensions of the acetone-dried mycelium.

Hydrolysis of hippuric acid by the suspensions was also shown by formation of glycine. This was shown to be present in the reaction mixtures by a chromatographic method, and it was also determined by the copper method.

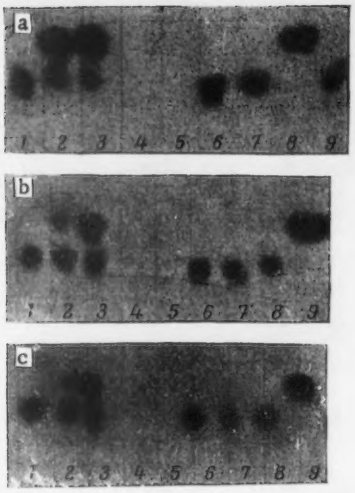

Fig. 1. Hydrolysis of hippuric acid by suspensions of Microsporum mycelium. a) Fresh Microsporum lanosum mycelium; b) acetone-treated Microsporum ferrugineum (Strain KK) mycelium; c) acetone-treated Microsporum ferrugineum (Strain K) mycelium. Solvent: n-butanol – acetic acid – water (4 : 1 : 1). 1) Sample taken before incubation; 2) the same, after 24 hr. incubation; 3) the same, after 48 hr. incubation; 4) control system (suspension + buffer) before incubation; 5) the same, after 48 hr. incubation; 6) control system (hippuric acid + buffer) before incubation; 7) the same, after 48 hr. incubation; 8) benzoic acid; 9) hippuric acid.

The results of glycine determination are presented in Table 2.

It is evident from the data of Table 2 that amino acid nitrogen increases in both the control systems (owing to autolysis of mycelium) and in the test systems, but that the rate of increase is much greater in the

TABLE 2

Amino Acid Nitrogen Content (mg %) of Reaction Systems Containing and not Containing Hippuric Acid

Species of mold	Suspension of	Control systems		Experimental systems	
		before incubation	after 48 hr	before incubation	after 48 hr
Microsporum lanosum	Fresh mycelium	8.4	16.1	8.4	23.8
Microsporum ferrugineum, Strain K	Fresh mycelium	4.2	7,8	5.6	12.5
	Acetone-dried mycelium	5.6	7.8	5.6	12.5
Microsporum ferrugineum, Strain KK	Fresh mycelium	4.9	7,8	4.9	14.0
	Acetone-dried mycelium	3,8	7,8	4.2	14.0

latter than in the former. This indicates that two processes are proceeding simultaneously in the test systems – breakdown of nitrogenous constituents of the mycelia (autolysis), with formation of amino acids, and hydrolysis of hippuric acid to benzoic acid and glycine. Our calculations show that 49% of the hippuric acid present initially had undergone hydrolysis in the presence of Microsporum lanosum mycelium, after 48 hr. of incubation, as compared with 30 – 39% with Microsporum ferrugineum. It should be noted that the data of Table 2 cannot be applied to the evaluation of the hippuricase activity of the different molds, as the amounts of mycelium present in the reaction mixtures were not the same. Nevertheless, these data complement the results of chromatography, and are evidence that fairly large amounts of hippuric acid undergo hydrolysis during 48 hr of incubation.

Our results thus show that both Fusarium and the Microsporum species have a fairly high hippuricase activity, and permit us to draw the conclusion that the poor growth of Microspora in hippuric acid media is ascribable to formation of benzoic acid, but not to their being deficient in hippuricase.

Our results also show that the hippuricase activities of suspensions of fresh and of acetone-dried mycelium are practically identical. This finding is in agreement with those of Smorodinzew [5], who showed that the hippuricase activities of fresh animal tissues did not exceed those of acetone- or ether-treated ones.

There are indications in the literature that hippuricase is firmly bound to the structural elements of animal tissues. Thus Smorodinzew [5] found that hippuricase (histozyme) was practically insoluble in water or in aqueous glycerol (1 : 1), and hence belongs to the class of endoenzymes, and that this enzyme, like certain other ones, exerts its action on the substrate in a medium in which it is itself insoluble. In view of these findings, we thought it necessary to determine whether the mycelial hippuricase was soluble in the phosphate buffer used in our experiments. The object of these experiments was to determine whether hippuric acid is hydrolyzed within the cells during the growth of the molds, or whether hydrolysis takes place in the medium.

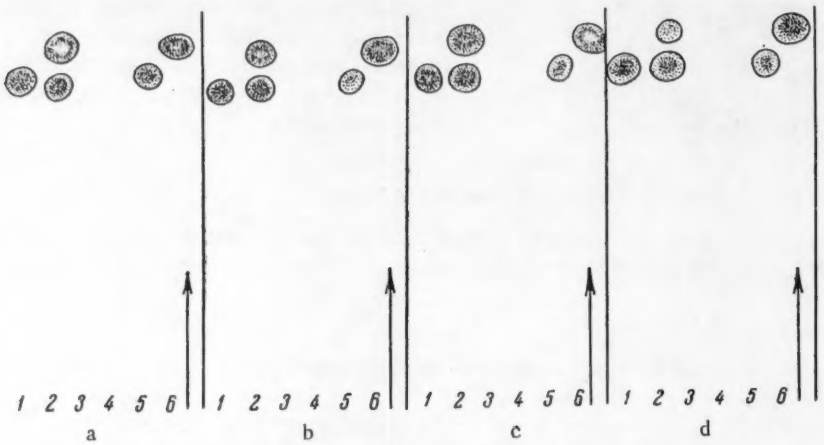

Fig. 2. Hydrolysis of hippuric acid by suspensions and extracts of Microsporum lanosum mycelium. a) Suspension of fresh mycelium; b) extract of fresh mycelium; c) suspension of acetone-dried mycelium; d) extract of acetone-dried mycelium. Duration of incubation of the mold: 111 days. Solvent system: n-butanol — acetic acid — water (4 : 1 : 1). 1) Test system before incubation; 2) the same, after 48 hr ; 3) control system (suspension or extract + buffer) before incubation; 4) the same, after 48 hr ; reference spots: 5) hippuric acid, 6) benzoic acid.

The experiments were performed on Microsporum lanosum mycelium, grown in glucose-peptone medium. Equal weights of the harvest of mycelium from a number of culture flasks were taken. One part was immediately ground up with phosphate buffer, while the other was treated with acetone, as described above. A certain amount of the suspension of fresh mycelium was tested at once, and the remainder was stored at 4 — 5° under a layer of toluene, with occasional shaking. The suspension was centrifuged after 24 hr , and the opalescent, pale yellow supernatant was tested for hippuricase activity. The amount of supernatant taken for the tests was equal to the volume of suspension of fresh mycelium taken before.

The acetone-treated mycelium was subjected to the same treatment as the fresh mycelium. The dry weight of this preparation amounted to 1.49 g.

The results of these experiments are shown in Fig. 2 (a, b, c, and d), from which it is evident that hippuric acid is hydrolyzed by extracts of fresh and acetone-dried mycelium as well as by the suspensions. This shows that the fungal hippuricase is readily soluble in M/15 phosphate buffer at pH 7.0. The conclusion might be drawn that when the molds are cultured under their natural conditions, hippuricase can diffuse out from the mycelium into the nutrient medium, where it can cause hydrolysis of hippuric acid.

4. Utilization of Benzoic Acid by the Molds

Our experiments on the growth of Fusarium in hippuric acid medium led us to think that benzoic acid

can be utilized by this species as a nutrient, at least when only very little glucose remains in the medium. This possibility was supported by Tauson's finding [9] that certain microorganisms are able to utilize benzoic and phenylacetic acids.

We therefore set up experiments designed to ascertain whether certain molds are capable of utilizing benzoic acid as a nutrient.

We took Fusarium, Microsporum lanosum, Microsporum ferrugineum (Strains K and KK), and Aspergillus for our experiments. The molds were inoculated into a nutrient medium containing 0.1% of glucose, 1% of benzoic acid, 0.1% of urea, and mineral salts and vitamins in the same concentrations as were taken for the hippuric acid medium. Inocula of each species were made into 10 flasks, each containing 45 ml of nutrient solution.

No growth of either strain of Microsporum ferrugineum was observed in this culture medium; Microsporum lanosum began to grow, but growth very soon ceased. Fusarium, on the other hand, grew very well in this medium, and had, by the 26th day, formed a fairly thick mycelial layer, which then took on the pink coloration characteristic of this mold. The yield of air-dry Fusarium mycelium amounted at this time to 95 mg per flask. The yield had not increased further by the 51st day, and had even somewhat diminished, as a result of autolysis. Aspergillus grew very slowly in this medium, and only a very small yield of mycelium was obtained after 10 days of incubation. The impression received was that the poor growth of this mold was due to lack of nutrient, since all the glucose was used up during the first few days. We noticed that the medium began to assume a dark reddish-brown color on the 8th to 9th day of incubation. This effect was not seen in Fusarium cultures in this medium. We cannot as yet explain the origin of this discoloration of the medium, although it may be supposed that it is related to some transformation of benzoic acid.

We analyzed the nutrient medium in which Fusarium had been grown. Benzoic acid was shown to be present by paper chromatography, and its content was determined gravimetrically.

TABLE 3

Analysis of Nutrient Medium Containing Benzoic Acid, in Which Fusarium Was Grown

Nutrient medium	pH	Glucose, mg-%	Total N, mg-%	NH_3-N, mg-%	Benzoic acid, mg-%	Yield of mycelium, mg per flask
Nutrient medium not inoculated (control)	7.11	92	47.0	none	988	—
Nutrient medium after 26 days of incubation	8.67	28	23.0	18.5	595	96
The same, after 51 days	9.0	none	15.4	9.0	none	86

The analytical results, given in Table 3, indicate a fall in the benzoic acid content of the medium during growth of Fusarium. There was a simultaneous fall in the glucose content. Thus the glucose content had fallen by 64 mg-%, and the benzoic acid content by 393 mg-%, by the 26th day of incubation. Although the absolute fall in benzoic acid content at that time was six times as great as of glucose, the latter is nevertheless utilized by the mold more rapidly than is benzoic acid. On the 51st day of incubation we could not find even traces of glucose or benzoic acid in the medium. These findings show that Fusarium is able to utilize benzoic acid as a source of carbon, and confirm our view that it is metabolized by this mold when it is grown in the hippuric acid medium.

The utilization of benzoic acid by Fusarium is connected with its enzymic oxidation. We cannot, on the basis of the data emerging from our experiments, say which enzyme systems are involved, or what is the mechanism of the reaction. It is clear that opening of the benzene ring must take place. It is possible that this is effected along one of the pathways envisaged by Tauson [9]. The straight-chain products resulting are oxidized eventually to carbon dioxide and water. This is shown by the copious evolution of carbon dioxide seen when the medium in which the mold has been grown is acidified with sulfuric acid.

We intend in a future research to investigate the nature of the intermediate products of degradation of benzoic acid in Fusarium and Aspergillus cultures.

The culture of molds in media containing benzoic acid has shown that the species concerned exhibit very marked differences in biochemical behavior; whereas benzoic acid is a poisonous substance with respect to Microspora, it is a nutrient to Fusaruim.

We can, on the strength of our experimental results, conclude that the poor growth of Microspora in a hippuric acid medium cannot be ascribed to their lack of hippuricase, but is due to the toxic action of benzoic acid liberated from hippuric acid.

SUMMARY

It has been found that a Fusarium of the Sporotrichiella group (Strain 1045) and Aspergillus niger show abundant growth when cultured in a medium containing hippuric acid as the sole nitrogen source. In the same medium, Trichoderma lignorum, Microsporum lanosum (Strain 2), and Microsporum ferrugineum (Strains K and KK) exhibit poor growth.

The Fusarium and the Microspora were found to possess fairly high hippuricase activity. The hippuricase of these molds can be readily extracted from the fresh or the acetone-dried mycelia by M/15 phosphate buffer at pH 7.0, and cannot be regarded as an endo-enzyme of these organisms.

The molds differ in their behavior in the presence of benzoic acid. Fusarium utilizes it as a source of carbon, and grows well in a medium containing benzoic acid as the chief source of carbon. Aspergillus shows only very poor growth in such a medium, while Microsporum species do not grow at all.

Since hippuricase is present in Microsporum species, their scanty growth in the hippuric acid medium is ascribed to the toxic effect of the benzoic acid liberated by the action of this enzyme.

The author takes pleasure in acknowledging his gratitude to Assistant E.S. Podgainaia and to Laboratory Technician N.M. Surikova for their assistance in fulfilling the experimental part of this research.

Received May 23, 1957

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STRUCTURE OF THE MANNAN OF DIPHTHERIA BACTERIA

O. K. Orlova

Chair of Biochemistry, Rostov-on-Don Medical Institute

The basic procedures now in use for the elucidation of the structure of polysaccharides are methylation, periodate oxidation, and enzymatic degradation. The methylation procedure, supplemented by the results of the periodate oxidation procedure, gives the fullest picture of the structure of polysaccharides.

In its classical form, the methylation procedure involves the following manipulations: methylation of the polysaccharide, hydrolysis of the methyl derivative, fractional distillation of the hydrolysis products, and identification of the fractions; these steps require a considerable amount of material (10 – 15 g). Such amounts of material are not readily available for the study of polysaccharides of bacterial origin.

The application of paper chromatography to the analysis of mixtures of methylated sugars, in conjunction with Hirst's method [1] for the determination of sugars after their chromatographic separation, permits the evaluation of the structure of polysaccharides, using only about 100 mg of the methylation product.

Replacement of fractional distillation by partition chromatography on silica gel, alumina, or cellulose powder has very greatly reduced the amount of material needed for the identification of methylated sugars in the form of their crystalline derivatives.

We were interested in the possibility of applying the methylation procedure, in conjunction with paper chromatography, to the investigation of the structure of the polysaccharide isolated by us in 1948 from diptheria bacteria, and classed by us as a mannan, since its sole hydrolysis product was D-mannose [2].

Polysaccharides of a similar constitution, also often called mannans, have been obtained from ivory nuts [3-5], yeast cells [6-8], waste products of mold fungi [9] and some aquatic plants [10].

Klages [4] investigated ivory nut mannan by the methylation procedure, and identified 2,3,4,6-tetramethyl- and 2,3,6-trimethyl-mannose among the hydrolysis products of the methylated polysaccharide. From his results this author concluded that ivory nut mannan is a straight chain of β-mannopyranose units in 1-4 linkage.

Mannocarolase, which is a polysaccharide formed from glucose by Penicillium charlesii, has been studied by Haworth [9]. Among the hydrolysis products of its methylation derivative (20 g) he found 2,3,4,6-tetramethyl- and 2,3,4-trimethyl-mannose, in the proportion of 1 : 5, together with an insignificant amount of 2,3-dimethyl-mannose. This author represented the polysaccharide as a chain of α-mannose units in 1 – 6 linkage.

Yeast cell mannan was first isolated and described by Salkovski [6] in 1894. Its structure was elucidated by Haworth [7,8], who used 20 g of the substance for this purpose. He found 2,3,4,6-tetramethyl-, 2,3,4-trimethyl-, and 3,4-dimethyl-mannose in equivalent amounts in the hydrolyzate.

The structure of the mannan of the seaweed Porphyra umbilicalis was elucidated by Jones [10] in 1950. By means of paper chromatography (using 100 mg of methylated mannan) and of chromatographic separation in a cellulose column (1 g of substance) he was able to identify 2,3,4,6-tetramethyl- and 2,3,6-trimethyl-mannose among the hydrolysis products.

Chromatographic methods have been applied in the investigation of a number of mannose-containing polysaccharides of plant and bacterial origin [11 – 15].

Our earlier work on the mannan of diphtheria bacteria showed that it is a low molecular weight polysaccharide. The molecular weight indicated by cryoscopic studies of solutions of acetylated mannan [16] is 4700, corresponding to 29 mannose residues per molecule.

The high dextrorotation of the mannan ($[\alpha]_{20} = 84°$) is evidence of the prevalence of α-links. The stability of the polysaccharide to acid hydrolysis is indicative of the pyranose structure of the units. Periodate oxidation [17] shows that the mannan is a highly ramified polysaccharide. The average chain length amounts to two mannose units.

The present research was undertaken with the object of achieving a more detailed picture of the molecular structure of this mannan.

EXPERIMENTAL

1. Methylation of Mannan

It is known from the literature that full methylation of most polysaccharides can only be achieved by repeating the operation several times, irrespective of the nature of the methylating agent or of the method employed. Inulin and levans can be more rapidly methylated than other polysaccharides, while on the other hand glycogen still possesses free hydroxyl groups after 20 methylations with dimethyl sulfate in the presence of concentrated alkali.

A number of workers have found that the number of methylations can be reduced by prior acetylation of polysaccharides.

As compared with other polysaccharides, mannans from different sources are more readily methylated. Thus, for example, ivory nut mannan gives a product containing 43.5% of methoxy groups after two methylations, and yeast cell mannan of the same methoxy group content is obtained after four methylations.

We used dimethyl sulfate as the methylating agent in our researches. The following methylation technique was adopted, on the basis of a large number of preliminary experiments.

One g of mannan dissolved in 3 ml of hot water was placed in a 3-necked flask. The flask was placed in a water bath, the temperature of which was maintained at 55-60° during methylation. We added dimethyl sulfate, at a rate of 1 drop every 10 seconds, and 60 ml of 30% NaOH, at a rate of 3 drops every 10 seconds, with energetic stirring of the reaction mixture. A nearly colorless mass of partial methylation product floated to the surface towards the end of the reaction. The residual solution was kept for 30 minutes in a boiling water bath, with continual stirring, and the methylation product that separated was collected and added to the first product. The product was soluble in cold water, but not in hot water or acetone.

The partially methylated mannan was dissolved in 5 ml of water, and subjected to methylation under the same conditions as before, except that small amounts of acetone were added from time to time. Methylated mannan did not separate out of the end of the second methylation. Some of the product floated to the surface at the end of the 30 minute treatment at 100°, and some settled to the bottom, together with salts. The product floating on the surface was collected, and the solution and precipitate were extracted repeatedly with acetone. Evaporation of the acetone extract of the solution gave only a very small yield of methylated mannan, much more of which was present in the acetone extract of the precipitate. All these methylated mannan fractions were pooled, and dissolved in acetone, and the acetone solution was treated with activated charcoal and filtered. The clear, colorless filtrate was poured into petroleum ether, and dried. The yield was 800 mg of air-dried product.

The white powder so obtained was soluble in acetone, chloroform, ethanol, and methanol, slightly soluble in cold water, and insoluble in hot water, benzene and petroleum ether. The optical rotation of a 1% solution of methylated mannan in acetone was $[\alpha]_{20} = 104°$. The methoxy group content was determined by Viebock and Brecher's modification [18] of Zeisel's method. We found a CH_3O content of 42.25%. We subjected 300 mg of our product to a third methylation. This yielded 70 mg of a product, having the same chemical properties as before, and a CH_3O content of 42.00%.

It thus appears that further methylation does not raise the methoxy group content of the product, but lowers the yield of methylated mannan.

2. Hydrolysis of the Methylated Mannan

Hydrolysis of methylated polysaccharides is usually effected in two stages – methanolysis in a 1.5 - 2% solution of dry HCl in absolute methanol, leading to the formation of methylglucosides of methylated sugars. This is followed by hydrolysis in N-HCl, and gives methylated sugars [7]. Direct hydrolysis with aqueous hydrochloric or sulfuric acids gives methylated sugars in one step [15]. Some authors first hydrolyze in glacial acetic acid or in anhydrous formic acid, and then conduct the hydrolysis in aqueous hydrochloric acid [19].

We applied various procedures to hydrolyze methylated mannan.

a) We placed 50 mg of methylated mannan in a 25 ml flask fitted with a condenser, added 10 ml of a 2% solution of HCl in absolute methanol, and heated the flask on a boiling water bath. The optical rotation of the solution was measured from time to time. Duration of hydrolysis (hours): 6, 8, 10, 12, 14. Angle of rotation (degrees): 0.39, 0.35, 0.34, 0.27, 0.27.

Equilibrium was attained after 12 hours. The mixture was made neutral with dry lead carbonate (neutralization with silver oxide gave no obvious advantage), and filtered. The residue of lead salts was washed on the filter with methanol. The filtrate and washings were evaporated to dryness in a porcelain dish, on the water bath. The residue of methylglucosides was returned to the hydrolysis flask, with 2.5 ml of 5% HCl, and hydrolysis was continued under the above conditions for 7 hr. The contents of the flask were made neutral with lead carbonate, and the filtrate was evaporated to dryness on the water bath. The methylated mannose derivatives were extracted from the dry residue with acetone, and the acetone solution was placed in a weighed beaker, and allowed to evaporate at room temperature. The yield amounted to about 30 mg.

b) We hydrolyzed 25 mg of methylated mannan in a tube containing 1 ml of N HCl for 10 hr. The contents of the tube were then neutralized with an aqueous suspension of silver oxide, and centrifuged. The centrifugate was evaporated down to dryness, and the residue was extracted with acetone, as above.

c) Hydrolysis was conducted as for b) above, but using sulfuric instead of hydrochloric acid, and neutralizing with dry barium carbonate.

3. Identification of Methylmannoses by Means of Paper Chromatography

Identification of methylated monosaccharides as their crystalline derivatives (anilides, lactones, etc.) requires their preliminary separation by means of fractional distillation, which requires considerable amounts of the products, or by means of partition chromatography on alumina columns [20], silica gel [21], or cellulose powder columns [22].

However, sufficiently trustworthy results may be obtained by means of paper chromatography, which does not require the preliminary separation of the products of hydrolysis of the methylated polysaccharide.

Any methylated monosaccharide can be identified from its R_g value: the ratio of the distance travelled by the given derivative to that travelled by tetramethylglucose under the same conditions and using the same solvent.

Tetramethylglucose was synthesized by Haworth's method [23], as modified by West and Holden [24]; the modified method gives higher yields, and is less time-consuming. However, we were unable to achieve the yields of tetramethylglucose from glucose claimed by these authors. This may have been due to our performing the synthesis on a much smaller scale than that used by West and Holden, in order to economize expenditure of dimethyl sulfate.

Tetramethylglucose was obtained in the form of characteristic needle-shaped crystals. However, the product was contaminated with a small admixture of trimethylglucose, which we were unable to remove by repeated recrystallizations. The methoxy group content of our product was 51.6%, which corresponds fairly closely to the theoretical value of 52.5%.

Of the solvent systems recommended in the literature for the separation of methylated monosaccharides, we used the following:

1. Butyl alcohol – acetic acid mixture: butyl alcohol – acetic acid – water (1 : 1 : 5).
2. Benzene – ethanol – water (167 : 47 : 15).

3. Butyl alcohol, not fully saturated with water.

4. Petroleum ether (b.p. 100 – 120°) – butyl alcohol (6 : 4 v/v).

5. Butyl alcohol – ethanol – water (41 : 11 : 19).

6. Butyl alcohol – ethanol – water – ammonia (40 : 10 : 49 : 1).

7. Methyl ethyl ketone, water saturated.

The best results were given by solvent mixtures 5 and 6. With these solvents tetramethylglucose gave an R_f value of 0.89.

The values of R vary according to the concentration, both for methylated and for free sugars. The chromatogram shown in Fig. 1 illustrates the dependence of the R_f value of tetramethylglucose on its concentration.

Chromatography of Hydrolyzates of Methylated Mannan

Spots of the hydrolyzates of methylated mannan, obtained by methods a, b, and c, were applied to a sheet of chromatographic paper (Leningrad Factory No. 2), dimensions 21 × 34 cm. The spots, each of which contained 1 mg of hydrolysis product, were applied alternately with tetramethylglucose spots, along a line 4 cm from the lower edge of the paper, the intervals between spots being 3.5 cm. Ascending chromatography was used, and developed with solvents 5 and 6, for a time of 18 – 20 hr. The chromatograms were then air-dried and sprayed with aniline hydrogen phthalate [25].

Well-defined chestnut-brown spots due to methylmannoses appeared on the chromatograms after drying for 5 minutes at 105°.

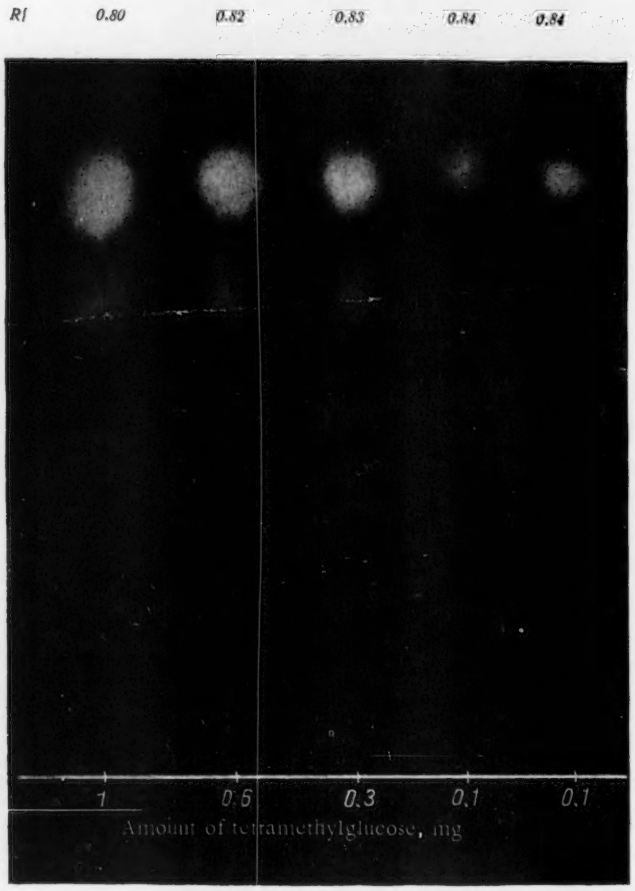


Fig. 1. Dependence of R_f value of tetramethylglucose on its concentration.

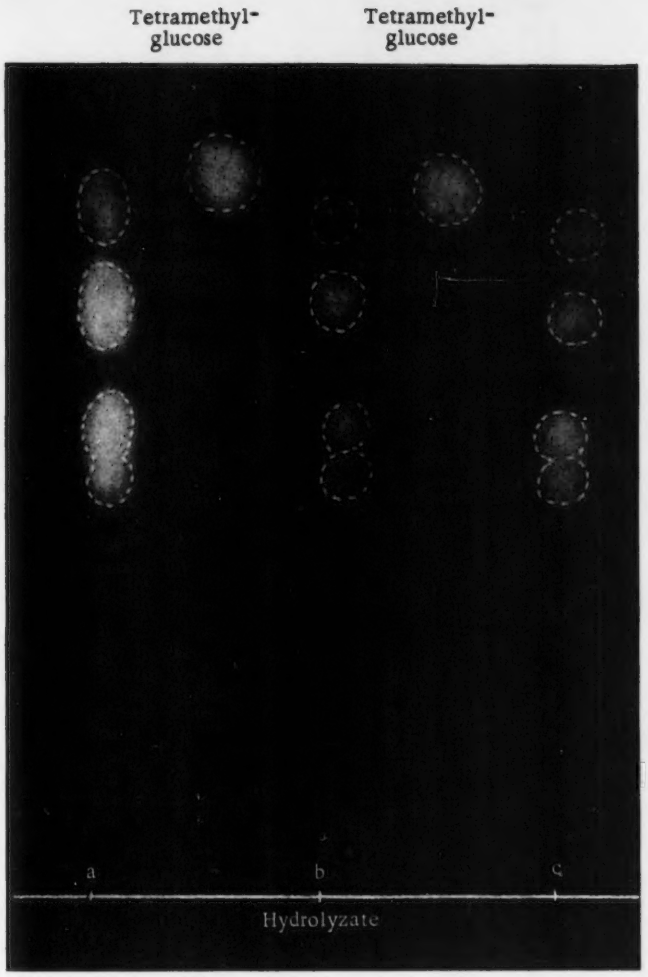


Fig. 2. Chromatogram of products of hydrolysis of methylated mannan by methods a, b, and c.

The results are presented in Fig. 2.

All three hydrolyzates gave the same picture: a spot at the level of the tetramethyl derivative, a spot at the level of the trimethyl derivative, and two confluent spots at the level of the dimethyl derivative. The two different dimethylmannoses present in the hydrolyzates appear to occur in equal amounts, as is shown by a comparison of the area and the intensity of coloration of the spots in a whole series of chromatograms. When large amounts of hydrolyzate are applied to the paper, very faint spots due to the monomethyl derivative can be distinguished. Similar results were obtained with a hydrolyzate of thrice-methylated mannan (Fig. 3).

Table 1 gives the R_g values found for methylmannoses (means of 6 determinations).

Hirst [1] gives the following values for methylated mannoses (solvent 6):

2,3,4,6-tetramethylmannose 0.96
2,3,6-trimethylmannose 0.81
3,4,6-trimethylmannose 0.80
2,3-dimethylmannose 0.54
3,4-dimethylmannose 0.58

Rafique [26], using the same solvent, found the following values:

2,3,4,6-tetramethylmannose 1.00
2,3,6-trimethylmannose 0.84
2,3,4-trimethylmannose 0.86
2,3-dimethylmannose 0.62
4-monomethylmannose 0.40

According to Andrews [12], R_g for 2,3,6-trimethylmannose is 0.89, with solvent system 5.

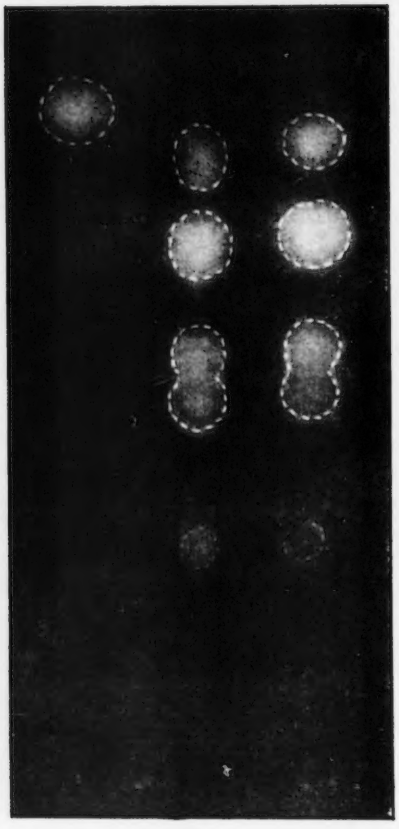


Fig. 3. Chromatogram of hydrolyzates of twice- and thrice-methylated mannan.

TABLE 1

R_g Values for Methylated Mannoses

Methyl derivative	Solvent mixture 5	Solvent mixture 6
Tetramethylmannose	0.97	0.94
Trimethylmannose	0.88	0.82
Dimethylmannose	a 0.73	a 0.66
	b 0.68	b 0.57
Monomethylmannose	0.49	0.38

It is not possible to establish the positions of the methoxy groups in the molecule of the mannose derivatives, obtained by hydrolysis of methylated mannan, on the basis of the R_g values alone. Thus, according to Rafique [26], the R_g values for 2,3,6- and 2,3,4-trimethylmannose are so close to each other as to render it impossible to distinguish between them on such evidence alone. The same applies to 2,3,6- and 3,4,6-trimethylmannose. The difference between the R_g values of 2,3- and 3,4-dimethylmannose is, however, sufficiently great to serve for the identification of these two isomers.

According to Hough [27], the use of dimethylaniline as a spray reagent enables conclusions to be drawn as to the location of the methoxy groups in the molecule. With this reagent, a purple coloration is obtained when a free hydroxyl group of a methylated aldohexose is situated at C_4.

It was necessary, in order to confirm this finding, to test the effect of this spray reagent on a spot of a methylated derivative of this type. As none could be found on the market, we were obliged to adopt the following solution.

Owing to the presence of 1 — 4 linkages in starch, the hydroxyl groups at the fourth carbon atom do not undergo methylation, so that hydrolysis of methylated starch should give considerable amounts of 2,3,6-trimethylglucose, which should afford a lilac coloration with dimethylaniline. We accordingly subjected a preparation of soluble starch to methylation (six-fold). We did not attempt to prepare a fully methylated derivative, as this was not essential for our purpose. The methylated starch was hydrolyzed according to method b, and the hydrolyzate was applied as a control spot, in the chromatography of methylated mannoses.

Spots of methylated mannan hydrolyzate, methylated starch hydrolyzate, and tetramethylglucose were applied to a sheet of chromatography paper of the usual size (21 x 34 cm).

Spraying with dimethylaniline gave one lilac spot from the methylated mannan hydrolyzate, at the level of dimethylmannose. The methylated starch hydrolyzate gave lilac spots at the level of trimethylglucose and dimethylglucose. Lilac spots were not seen at the level of tetramethylglucose. The spots were outlined in pencil, and the same chromatogram was sprayed with aniline hydrogen phthalate reagent. Additional spots then appeared, due to tetramethyl-, trimethyl-, and dimethyl-mannose, the last of which was situated above the spot shown up by dimethylaniline after the first spraying. The methylated starch hydrolyzate gave a spot due to tetramethylglucose, and the tetramethylglucose spot gave the usual intense coloration.

The results are illustrated by the chromatogram of Fig. 4.

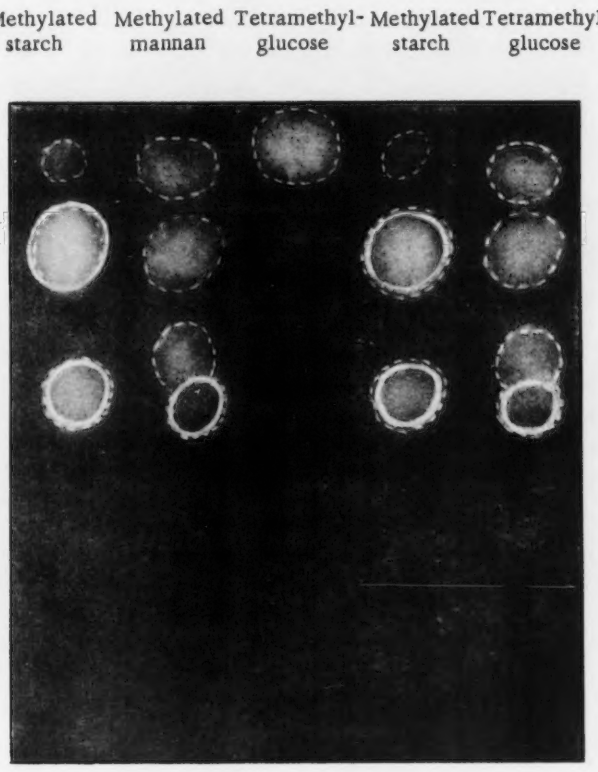


Fig. 4. Chromatogram of hydrolyzates of methylated mannan and starch, sprayed successively with dimethylaniline and aniline hydrogen phthalate. Continuous lines encircle the spots shown up by dimethylaniline, and broken lines those shown up by aniline hydrogen phthalate.

The following conclusions may be drawn from the results of successive spraying with the two reagents, in conjunction with the R_g values.

1. The tetramethylmannose present is 2,3,4,6-tetramethylmannose (R_g = 0.94).

2. The trimethylmannose (R_g = 0.82) is either 2,3,4- or 3,4,6- trimethylmannose. We think it is more likely to be the former than the latter isomer. Were it to be the former it would imply the existence of 1 – 6 linkages in the main chain and its branches, while the latter would imply 1 – 2 linking. Periodate oxidation [17] gives one molecule of formic acid per two mannose residues. Were the residues to be linked through the 1 – 6 carbon atoms we should expect one molecule of formic acid per 1.3 mannose residues, with the ratio of components indicated by the subsequent experiments. If the residues are connected by 1 – 2 linkages, one molecule of formic acid should be liberated per four mannose residues. The results found are more in accordance with

the former variant. The deviation from the theoretical result is obviously a result of incomplete oxidation, since we used potassium instead of sodium periodate for this purpose.

3. If we assume that the trimethylmannose is 2,3,4-trimethylmannose, then the dimethylmannose reacting with dimethylaniline must be 2,3-dimethylmannose (R_g = 0.57). As for the second dimethylmannose (R_g = 0.66), we have no evidence, direct or indirect, enabling us to choose between the 2,4- and 3,4-dimethylmannose structures.

The presence of two dimethylmannoses points to the existence of branching from the main chain at different points. The presence of 3,4- or 2,4-dimethylmannose may have been a result of incompleteness of methylation, or it may have resulted from elimination of methoxy groups from trimethyl- or tetramethyl-mannose. Against the former interpretation is the observation that the spot due to the second dimethylmannose (2,4- or 3,4-) appears for hydrolyzates of both twice- and thrice-methylated mannan at about equal intensities. The presence of this spot on the chromatograms of methylated mannan hydrolyzates obtained by different methods is an argument against the latter interpretation.

4. Quantitative Determination of Methylmannoses Separated by Means of Paper Chromatography

Chromatographic separation of methylmannoses, with the object of their subsequent determination, was done on 21 x 45 cm sheets, the solvent front being allowed to travel about 40 cm, which required a time of 42-45 hr (solvent system 6).

Five hydrolyzate spots were applied to each chromatogram (each 0.1 ml of hydrolyzate containing about 4 mg of methylmannoses). After drying, we cut out the middle and the lateral strips, and sprayed them with aniline hydrogen phthalate, superimposed them on the untreated strips, and marked the locations of the spots corresponding with the various methyl derivatives. The dimethylmannoses were determined together. The areas of the paper corresponding with the spots were cut out, as well as equal areas of paper from their vicinity, to serve as controls. The pieces of paper were folded several times, and left for an hour with 4 ml of hot water in 10 ml beakers. The extract was then poured off, and the paper washed twice with 1 ml portions of water; the extract + washings were placed in test-tubes (2.5 x 22.5 cm) with ground glass stoppers. To each tube we added 2 ml of buffer solution at pH 11.3 (25 ml of 0.1 N Na_2HPO_4 + 8.7 ml of 0.1 N NaOH, followed by water to 50 ml), and 1 ml of 0.1 N iodine solution, after which the stoppers were wetted with 10% KI solution, and tightly inserted. The tubes were kept in the dark at room temperature for 20 hr, after which the stoppers were washed with water, and the washings were added to the solution, which was made up to 25 ml, and acidified with 2 ml of 2N H_2SO_4. The iodine liberated was then titrated with 0.01 N thiosulfate. The volume of thiosulfate used in the titration is equivalent to the methylmannose content of the solutions, and the ratios of the volumes of thiosulfate used for titration are equal to the molecular proportions of the various methylmannoses present in the hydrolyzates [28].

TABLE 2

Reducing Power of Methylated Mannoses

Methylated sugar	Volume of 0.01 N thiosulfate, in ml		
	Experiment 1	Experiment 2	Experiment 3
Tetramethylmannose	0.45	0.40	0.45
Trimethylmannose	0.80	0.85	0.90
Dimethylmannoses (combined)	0.45	0.45	0.40
Molecular proportions of components	1:2:1	1:2:1	1:2:1

Preliminary experiments established the possibility of determination of the methylmannoses without their preliminary extraction. The folded pieces of paper containing the appropriate spots of methylmannoses were placed directly in the titration tubes. The procedure following this was the same as before.

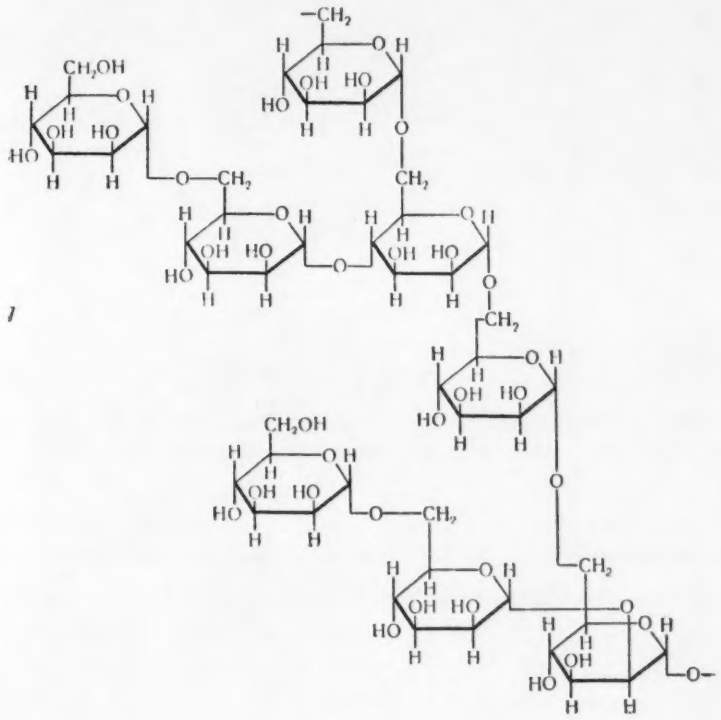
Both methods gave identical results for the proportions of the components of the hydrolyzates (Table 2).

Thus, ignoring the very small content of monomethylmannose in the hydrolyzates, we found that tetramethylmannose, trimethylmannose, and dimethylmannose were present in the molecular proportions 1 : 2: 1. If we take the contents of the two dimethylmannoses as being equal, the proportions are 2 : 4 : 1 : 1.

On the basis of our qualitative and quantitative findings relating to the components of the hydrolyzate of methylated mannan, we consider that two variants of its molecular structure might be formulated.

Variant 1: 2,3,4,6-tetramethylmannose
2,3,4-trimethylmannose
2,3-dimethylmannose
3,4-dimethylmannose

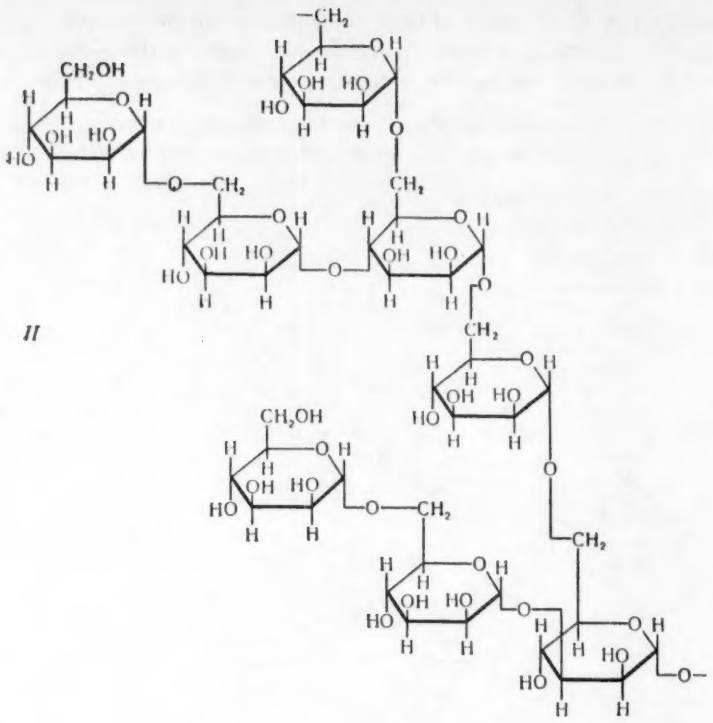
in the proportions 2 : 4 : 1 : 1, which agrees with Formula I.



Variant 2: 2,3,4,6-tetramethylmannose
2,3,4-trimethylmannose
2,3-dimethylmannose
2,4-dimethylmannose

in the proportions 2 : 4 : 1 : 1, which corresponds to Formula II.

More trustworthy data could be derived from the identification of the methylmannoses (as anilides and lactones) after their separation by means of fractional distillation or of column chromatography.



II

SUMMARY

Methylation of the mannan of diphtheria bacteria by dimethylsulfate yields a product with a maximum of 42.25% of methoxy-groups after the second methylation. No increase in methoxy-groups was obtained by further methylation.

Methylated mannan is easily soluble in acetone, chloroform and in methyl and ethyl alcohol, but insoluble in hot water, benzene or petroleum ether. Its specific rotation equals 104° (1% acetone solution).

Among the products of methylated mannan the following have been identified by paper chromatography: tetramethylmannose (R_g = 0.94), trimethylmannose (R_g = 0.82), dimethylmannose (R_g = 0.66 and R_g = 0.57).

By developing the chromatograms of the hydrolyzates of methylated mannan successively with two chemicals (dimethyl aniline and aniline hydrophthalate) the following compounds have been revealed 2 : 3 : 4 : 5 : 6-tetramethylmannose, 2 : 3 : 4- or 3 : 4 : 6-trimethylmannose, 2 : 3- or 3 : 6-dimethylmannose, 2 : 4-, 3 : 4- or 4 : 6-dimethylmannose. A quantitative assay of these four components shows a ratio of 2 : 4 : 1 : 1.

Two alternative structures of the mannan of diphtheria bacteria are suggested by the above evidence as well as by the data of preceding studies.

a. The principal chain of mannan contains 1-6 bonds. From every second mannose residue of the principal chain side chains consisting of two mannose residues are branching being alternatively attached by 1-4 and 1-2 bonds. In the side chains the mannose residues are connected to one another by 1-6 bonds.

b. In distinction to the foregoing structure, the side chains are alternatively attached by 1-4 and 1-3 bonds.

Received July 9, 1957

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EFFECT OF CARNOSINE ON THE REACTION OF GLYCOLYTIC OXIDATION-REDUCTION COUPLED WITH PHOSPHORYLATION

N. K. Nagradova

Chair of Biochemistry of Animals, M. V. Lomonosov Moscow State University

A number of experiments have been devoted to the study of the stimulating effect of carnosine on the reaction of glycolytic oxidation-reduction coupled with phosphorylation [1,2]. Addition of carnosine to a dialyzed muscle extract leads to a fall in the fructose diphospate content, and to enhanced formation of phosphoglyceric acid. We showed, in our previous publication [3], that this effect of carnosine is only manifested when the dialyzed extract is incubated with phosphate buffer (0.12 M). When we took bicarbonate or glycine buffers containing 0.0004 M arsenate (which allowed the oxidation-reduction reaction to proceed in the absence of phosphate), addition of carnosine did not cause any rise in phosphoglyceric acid production.

In systems containing crystalline aldolase and phosphoglyceraldehyde dehydrogenase, in the absence of conditions necessary for the transfer of phosphate residues to the adenylic acid system, enhanced production of phosphoglyceric acid did not occur after addition of carnosine. We thus concluded that the effect of carnosine on the reaction of glycolytic oxidation-reduction in dialyzed muscle extract is a result of the stimulation of transfer of phosphate from 1,3-diphosphoglyceric acid to ADP*.

The object of the present research was to achieve this reaction in an isolated system, and to examine the possibility of its activation by carnosine. While this research was in progress we found that, under certain definite conditions, carnosine, as well as some other substances, affected the reaction of formation of 1,3-diphosphoglyceric acid, which made it necessary for us to examine this effect in greater detail.

EXPERIMENTAL

In our previous publication [3] we reported the effect of carnosine on the reaction of formation of phosphoglyceric acid when fructose diphosphate is added to dialyzed rabbit muscle extract, using creatine as an acceptor of phosphate from ATP. A similar effect of carnosine was seen when oxidation of phosphoglyceraldehyde took place independently of the aldolase and creatine phosphokinase reactions. In experiments in which dialyzed muscle extract was taken as the enzyme system, with a mixture of triose phosphates as the substrates, and with ADP as phosphate acceptor, we found that addition of carnosine caused an increased production of phosphoglyceric acid and ATP (Table 1).

Minced rabbit muscle was extracted with 1 $^1/_2$ volumes of cold water for 30 min. The extract was centrifuged, and the supernatant was dialyzed against tap water for 6 - 7 hr. Triose phosphates (mixtures of equal amounts of phosphoglyceraldehyde and dihydroxyacetone phosphate) were prepared enzymatically from fructose diphosphate by Meyerhof's method [4]; the concentration of the solutions was derived from measurements of the amount of inorganic phosphate eliminated by hydrolysis with N alkali [5]. ADP (88% pure) was obtained by the action of myosin on ATP.

Phosphoglyceric acid was determined by the resorcinol method [5]. ATP and ADP were separated chromatographically, and their content was estimated by determination of adenine [6].

* Abbreviations used: ATP is adenosinetriphosphate, ADP is adenosinediphosphate, DPN is diphosphopyridine nucleotide, DPNH is the reduced form of DPN, and PGA is phosphoglyceraldehyde.

TABLE 1

Effect of Carnosine on the Reaction of Oxidation of Phosphoglyceraldehyde

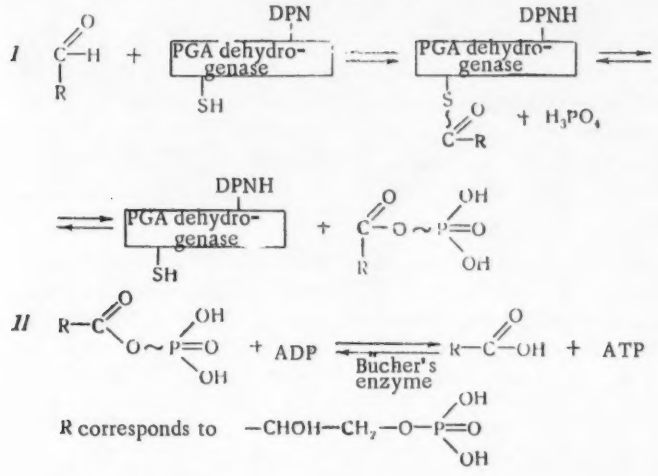
Substance under analysis (μ moles present in the systems)	Incubation time, in min					
	5		15		30	
	without carnosine	with carnosine	without carnosine	with carnosine	without carnosine	with carnosine
Phosphoglyceric acid	0.9	–	1.5	2.3	2.3	3.3
ATP	0.8	1.0	1.2	1.6	1.7	2.4

The composition of the systems before incubation was: triose phosphates – 34 μ moles in 1 ml, Na pyruvate – 18 μ moles in 1 ml, ADP – 9.2 μ moles in 1 ml, NaF – 0.05 M, phosphate buffer – 0.12 M, pH 7.4, dialyzed muscle extract – 0.15 ml. The systems had a volume of 1.45 ml. Carnosine was dissolved in buffer solution containing all the components of the reaction, to give a concentration of 500 mg per 100 ml, i.e., about 0.022 M. The pH of the systems with and without carnosine was equalized, checking the pH by means of a glass electrode.

The reaction was initiated by adding enzyme extract in buffer solution. After 5, 15, and 30 min. of incubation at 20° protein was precipitated by adding trichloroacetic acid to a final concentration of 2.5%, and ATP and phosphoglyceric acid were determined in the protein-free centrifugate.

In order to be able to study the second phase of the reaction of oxidation of PGA (transfer of phosphate residue from 1,3-diphosphoglyceric acid to ADP) separately from the first phase, catalyzed by PGA dehydrogenase (see formulation), we needed to have at our disposal an adequate supply of 1,3-diphosphoglyceric acid.

Formulation of the reaction of oxidation of phosphoglyceraldehyde, coupled with phosphorylation



There are indications in the literature [7] that if PGA dehydrogenase is incubated with phosphoglyceraldehyde and phosphate, under conditions ensuring re-oxidation of the DPNH formed, considerable amounts of 1,3-dephosphoglyceric acid may accumulate. We investigated the rate of formation of 1,3-diphosphoglyceric acid in systems containing crystalline preparations of aldolase, PGA dehydrogenase, and lactic dehydrogenase, as well as fructose diphosphate and pyruvic acid.

Crystalline preparations of aldolase, PGA dehydrogenase, and lactic dehydrogenase were made from a single portion of rabbit muscle by the method of Beisenherz et al. [8]. The suspensions of enzyme crystals in ammonium sulfate solutions of the appropriate concentrations were stored at low temperature. When required, the suspensions were centrifuged at 16 000 g at 0°, and the crystals were dissolved in cooled redistilled water.

A fixed volume of dissolved protein was taken in the experiments. The protein concentration of the solutions was determined by a method based on the biuret reaction [8]. DPN was prepared and purified by Clark's method [9]. The purity of the preparation, as tested enzymatically, amounted to 60%. Fructose diphosphate (70% pure) was added as the Na salt.

1,3-Diphosphoglyceric acid was determined by the method of Lipmann and Tattle [10], involving treatment of the solutions with hydroxylamine, followed by colorimetric determination of the hydroxamic acid liberated. The experimental procedure was as follows. Accumulation of diphosphoglyceric acid took place during incubation of the systems containing fructose diphosphate, aldolase, PGA, and orthophosphate. The reaction began in all cases with addition of PGA dehydrogenase. Samples were taken at fixed time intervals, and were added to a mixture of 1 ml of 2 N hydroxylamine at pH 6.4 and 1 ml of 0.1 N acetate buffer at pH 5.4. Water was added to 3 ml, and the solutions were deproteinized after 10 min. by addition of 1 ml of 12% CCl_3COOH. Color development took place on addition of 1 ml of 2.7 N HCl and 1 ml of 5% $FeCl_3$ in 0.1 N HCl. Optical densities were measured in a photocolorimeter, and the amount of 1,3-diphosphoglyceric acid was read from a standard curve based on measurements with succinic anhydride.

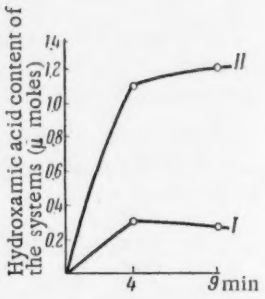


Fig. 1. Effect of addition of lactic dehydrogenase and pyruvic acid on the yield of 1,3-diphosphoglyceric acid. Composition of systems: I. Triose phosphates) 7.4 μ moles in 1 ml of 0.05 M $NaHCO_3$ and 0.15 M KH_2PO_4, DPN) 0.06 μ moles in 1 ml, PGA dehydrogenase 0.05 mg per ml; II) the same, but with addition of 36 μ moles of Na pyruvate per ml and of 0.1 mg per ml of lactic dehydrogenase. Volume of systems: 1.85 ml; pH 8.3; reaction temperature 19°.

It is evident from the reaction scheme that, apart from 1,3-diphosphoglyceric acid, an acyl thio-ester of PGA dehydrogenase is formed, which could also give rise to hydroxamic acid when treated with hydroxylamine [11]. However, the amount of hydroxamic acid which might have been formed from this source is determined by the PGA dehydrogenase content of our systems, which varied from 0.03 to 3.0 mg; it may therefore be taken that the greater part of the hydroxamic acid formed, originated from 1,3-diphosphoglyceric acid. Accordingly, we shall assume that the amount of hydroxamic acid found is a direct measure of the 1,3-diphosphoglyceric acid content of the systems.

The rate of oxidation of phosphoglyceraldehyde is illustrated by the curves of Fig. 1. When transfer of hydrogen from DPNH to pyruvate does not occur (Curve I) only small amounts of hydroxamic acid are formed. Addition of lactic dehydrogenase and Na pyruvate to the system increases the reaction velocity, and the yield of hydroxamic acid rises considerably, since the PGA dehydrogenase is able to release both the oxidized PGA residue (by phosphorolysis) and the DPNH (by reaction with pyruvate), thus raising its turnover.

1,3-Diphosphoglyceric acid is a very unstable substance; we found that raising the reaction temperature from 18 to 37° causes quite considerable decomposition of this product – whereas at 18° the rate of accumulation of 1,3-diphosphoglyceric acid falls off by the end of 30 min , and equilibrium is achieved by the end of 45-60 min., at 37° the rate of decomposition exceeds that of production at the end of 30 min , while after 60 min. the greater part of the 1,3-diphosphoglyceric acid formed during the reaction has undergone decomposition (Table 2). In view of this finding, all our further experiments were conducted at 18 - 21°.

Having found the necessary conditions for accumulation of 1,3-diphosphoglyceric acid in the systems, we proceeded to the investigation of the second stage of the reaction, viz., transfer of a phosphate residue to ADP. With this object, we added iodoacetate to a sample of the system in which 1,3-diphosphoglyceric acid had been allowed to accumulate, so as to inactivate PGA dehydrogenase.

It can be seen from Fig. 2 that addition of iodoacetate to a final concentration of 0.003 M, after 10 minutes of incubation, prevents further accumulation of 1,3-diphosphoglyceric acid (Curve II), while when it is added at the beginning of the reaction formation of this product is totally inhibited (Curve III). Inactivation

of PGA dehydrogenase by iodoacetate is not associated with inactivation of the enzyme catalyzing the reaction between 1,3-diphosphoglyceric acid and ADP Bücher enzyme). The accumulated 1,3-diphosphoglyceric acid can therefore serve as a substrate for the reaction of transfer of a phosphate residue to ADP.

TABLE 2

Effect of Temperature on Accumulation of 1,3-Diphosphoglyceric Acid

Composition of systems: fructose diphosphate) 25 μ moles in 1 ml, aldolase) 0.26 mg per ml, Na pyruvate) 6 μ moles in 1 ml, DPN) 0.06 μ moles in 1 ml, KH_2PO_4) 0.02 M, pyrophosphate buffer) 0.01 M, lactic dehydrogenase) 0.8 mg per ml, PGA dehydrogenase 0.23 mg per ml. Volume 3.2 ml; pH 7.6 - 7.7. μ Moles of hydroxamic acid found in the systems.

Temperature, °C	Time of incubation, min		
	30	45	60
18	6.0	6.4	6.7
37	5.2	3.7	3.0

Our preparation of Bücher enzyme was a dialyzed muscle extract previously treated with iodoacetate.

It is evident from Fig. 3 that the addition of such an extract to a system which had been incubated for 30 min. arrested the further accumulation of 1,3-diphosphoglyceric acid (Curve I). When ADP was added simultaneously there was a sharp fall in the 1,3-diphosphoglyceric acid content, due to transfer of phosphate residues to ADP. These experimental conditions appeared to us to be suitable for the investigation of the effect of carnosine on the process of transfer of phosphate residues from 1,3-diphosphoglyceric acid to ADP.

We were unable, in a series of experiments carried out under various conditions, to observe any effect of carnosine on the velocity of dephosphorylation of 1,3-diphosphoglyceric acid in the presence of ADP and dialyzed muscle extract (Table 3).

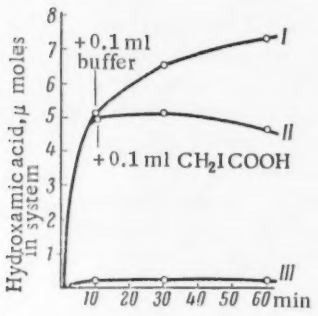


Fig. 2. Effect of iodoacetate on accumulation of 1,3-diphosphoglyceric acid.

Composition of the systems: fructose diphosphate) 19 μ moles in 1 ml, aldolase) 0.3 mg per ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 5 μ moles in 1 ml, NaF) 0.045 M, $MgSO_4$) 0.0005 M, glycine buffer) 0.06 M, KH_2PO_4) 0.018 M, lactic dehydrogenase) 0.95 mg per ml, PGA dehydrogenase) 0.4 mg per ml. Volume of the systems: 3.7 ml; pH 7.7; incubation temperature 20°. Curve I: incubation without iodoacetate, Curve II: iodoacetate added after 10 min. of incubation, Curve III: iodoacetate added initially, to a concentration of 0.003 M.

From the results of this series of experiments we drew the conclusion that the effect of carnosine on the overall process I + II (see formulation) cannot be ascribed to activation of the second step, catalyzed by Bücher's enzyme. The velocity of this reaction is very high; when undiluted dialyzed enzyme extract is taken it proceeds too swiftly to permit a study of its kinetics. In the experiments described in Table 3 we used enzyme solutions which had been diluted 70-fold.

Since our results did not support the hypothesis that the stimulatory effect of carnosine is exerted on the second stage of the reaction, we were obliged to revert to the closer investigation of its first stage, viz., the oxidation of phosphoglyceraldehyde.

Both fructose diphosphate (taken together with aldolase) and triose phosphate mixtures were taken as substrates for the reaction. The progress of the reaction of oxidation of PGA was followed from the rise in the content of acyl derivatives (acyl-enzyme compound, 1,3-diphosphoglyceric acid) with time; these products were converted into hydroxamic acid, which was determined colorimetrically. In some of the experiments we

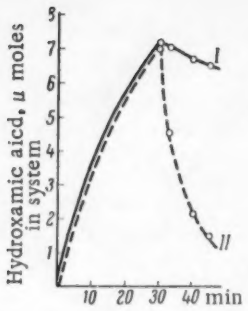


Fig. 3. Accumulation of 1,3-diphosphoglyceric acid, and its reaction with ADP.

Composition of systems: fructose diphosphate) 28 μ moles in 1 ml, aldolase) 0.3 mg per ml, DPN) 0.075 μ moles in 1 ml, Na pyruvate) 7 μ moles in 1 ml, NaF) 0.05 M, $MgSO_4$) 0.0005 M, glycine buffer) 0.03 M, KH_2PO_4) 0.023 M, lactic dehydrogenase 1.3 mg per ml, PGA dehydrogenase) 0.5 mg per ml. Volume of systems 2.7 ml; pH 7.7, incubation temperature 20°. At the end of 30 min. we removed 0.5 ml samples of the systems for the determination of 1,3-diphosphoglyceric acid as hydroxamic acid, and we added: to system I, 0.5 ml of enzyme extract and 0.5 ml of 0.1 M glycine buffer; to system II, 0.5 ml of enzyme extract and 0.5 ml of ADP solution (10.7 μ moles). Final concentration of iodoacetate after addition of enzyme extract – 0.003 M. Further samples were withdrawn for hydroxamic acid determination at 33, 40, and 45 min.

TABLE 3

Effect of Carnosine on the Reaction of Transfer of Phosphate from 1,3-Diphosphoglyceric Acid to ADP

Composition of systems: 1) fructose diphosphate) 25 μ moles in 1 ml, aldolase) 0.23 mg per ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 5 μ moles in 1 ml, $MgSO_4$) 0.0005 M, glycine buffer) 0.06 M, KH_2PO_4) 0.018 M, lactic dehydrogenase) 0.7 mg per ml, PGA dehydrogenase) 0.4 mg per ml, carnosine) 0.022 M (500 mg-%). Volume of the systems) 1.85 ml; pH 7.4; temperature 19°. After 30 min. we withdrew 0.3 ml portions for determination of 1,3-diphosphoglyceric acid as hydroxamic acid, and added 0.5 ml of ADP solution (10.7 μ moles) and 0.2 ml of enzyme extract (before incubating it with iodoacetate we diluted it 70-fold). 2) Fructose diphosphate) 23 μ moles in 1 ml, aldolase) 0.8 mg per ml, Na pyruvate) 18 μ moles in 1 ml, $MgSO_4$) 0.0005 M, $NaHCO_3$) 0.05 M, KH_2PO_4) 0.15 M, lactic dehydrogenase) 0.22 mg per ml, PGA dehydrogenase) 0.6 mg per ml, carnosine) 0.0088 M (200 mg-%). Volume of the systems) 1.8 ml; pH 8.1; temperature 19°. After 40 min. we withdrew 0.2 ml portions for determination of 1,3-diphosphoglyceric acid as hydroxamic acid, and added 0.2 ml of enzyme extract (prepared as in System I) and 0.5 ml of ADP solution (7.0 μ moles).

Hydroxamic acid content of the systems, in μ moles

System	Incubation time, min				
	30	33	40	50	60
	System 1, glycine buffer				
With carnosine	4.0	3.6	2.6	1.8	1.3
– carnosine	3.8	3.5	2.5	1.8	1.2
	System 2, phosphate buffer				
	40	43	56	70	85
With carnosine	4.3	3.6 3.0	2.2	1.5	0.8
– carnosine	4.4	3.6 3.0	2.1	1.4	0.7

applied a spectrophotometric method to the study of reaction rates (from the rise in optical density of the systems at 340 mμ, due to reduction of DPN).

We were unable to detect any effect of carnosine on the rate of increase in 1,3-diphosphoglyceric acid or DPNH contents of systems buffered with glycine solutions (Fig. 4).

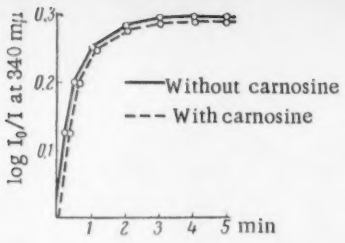


Fig. 4. Effect of carnosine on oxidation of phosphoglyceraldehyde in systems buffered with glycine.
Composition of systems: triose phosphates 6 μ moles in 1 ml, DPN) 0.11 μ moles in 1 ml, glycine buffer) 0.05 M, KH_2PO_4) 0.12 M, PGA dehydrogenase) 0.0018 mg per ml, carnosine) 0.044 M (1000 mg-%). Volume of systems) 2.7 ml; pH 8.1; reaction temperature 20°. Depth of cuvette) 1 cm.

TABLE 4.

Effect of Dilution of PGA Dehydrogenase Solution on its Activity in Systems Containing Phosphate and Glycine Buffers
Composition of systems: fructose diphosphate) 27 μ moles in 1 ml, aldolase) 0.19 mg per ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 18 μ moles in 1 ml, 0.12 M phosphate buffer, or 0.06 M glycine buffer containing 0.03 M KH_2PO_4, lactic dehydrogenase) 0.21 mg per ml, PGA dehydrogenase) various amounts. Volume of systems) 1.85 ml; pH 7.6; temperature 19°. The hydroxamic acid content was determined (in μ moles) in each system after 30 min of incubation.

mg PGA dehydrogenase added	1.59	0.79	0.38	0.185	0.048
μ Moles hydroxamic acid found:					
in phosphate buffer	3.75	2.9	2.25	1.75	0.0
in glycine buffer	3.5	3.3	3.2	2.9	2.1

Bearing in mind that in our experiments with dialyzed enzyme extract, the effect of carnosine was only seen in phosphate-buffered systems, we proceeded to examine the effect of the buffer on the course of the reaction in isolated enzyme systems. Parallel incubation of systems with phosphate or glycine buffer showed that the rate of formation of 1,3-diphosphoglyceric acid was much lower with phosphate buffer (Fig. 5, A). When we added carnosine to the phosphate-buffered systems we observed increased formation of hydroxamic acid, whereas hardly any effect was seen with glycine-buffered systems. The impression was that carnosine restores the activity of the dehydrogenase, which had been lowered in the phosphate systems, but was unaffected in the glycine systems.

Such an effect of carnosine could only be seen under certain definite conditions. Thus, if we raised the PGA dehydrogenase content of the systems (in our experiments to 0.8 - 1.7 mg per ml) the reaction proceeded at the same rate in both glycine- and phosphate-buffered systems, and addition of carnosine was without effect (Fig. 5, B).

At a concentration of 0.12 M, phosphate inhibits the reaction catalyzed by PGA dehydrogenase when the enzyme concentration falls below certain limits. When the enzyme protein concentration is high (about 1 mg in 1 ml, in our experiments), the turnover of the enzyme is small, and phosphate inhibition is practically absent. As the concentration of the enzyme is lowered, the composition of the systems being otherwise unaltered, the inhibitory effect of phosphate rises progressively.

Table 4 presents the results of an experiment in which phosphate- and glycine-buffered systems were incubated at the same time. The reaction rate curves showed that equilibrium was achieved within 30 min. of incubation. It is evident from Table 4 that an 8.6-fold lowering of dehydrogenase concentration of glycine-buffered systems had little effect on the yield of 1,3-diphosphoglyceric acid, whereas an equal dilution of the enzyme in phosphate-buffered systems caused a considerable fall in the yield. A further four-fold dilution of the enzyme led to its total inactivation in phosphate-buffered systems.

As is evident from Fig. 5, the effect of carnosine is to protect PGA dehydrogenase from the inhibitory action of phosphate. Addition of carnosine restores the activity of PGA dehydrogenase, and raises the reaction

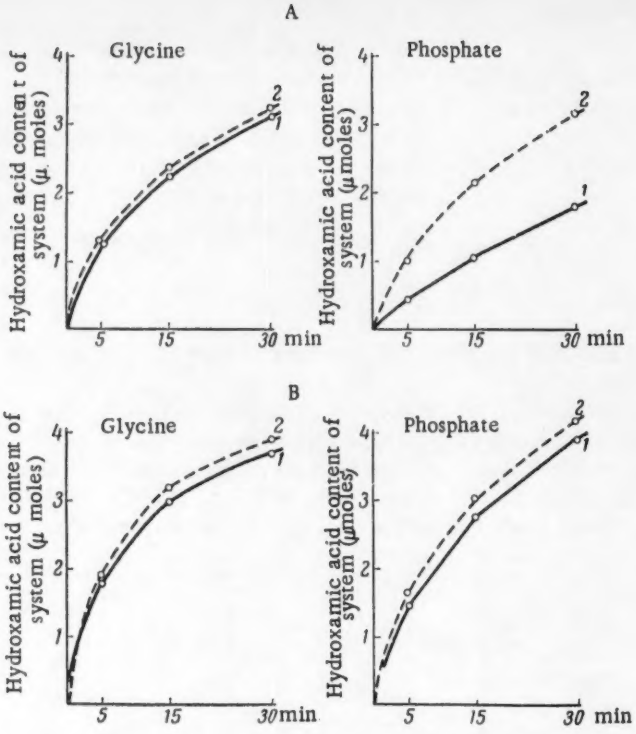
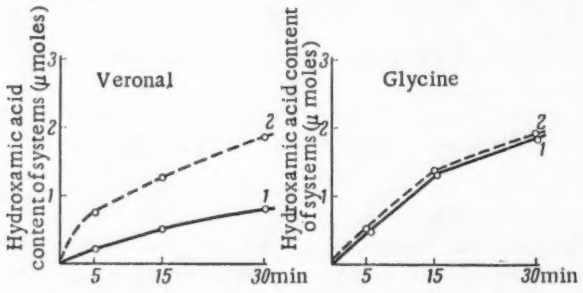


Fig. 5. Effect of carnosine on the reaction of oxidation of phosphoglyceraldehyde, under various conditions. Composition of systems: fructose diphosphate) 27 μ moles in 1 ml, aldolase) 0.19 mg per ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 18 μ moles in 1 ml, lactic dehydrogenase) 0.21 mg per ml, PGA dehydrogenase: A) 0.28 mg per ml; B) 1.7 mg per ml. 0.12 M phosphate buffer or 0.06 M glycine buffer containing 0.03 M KH_2PO_4. Carnosine) 0.22 M (500 mg-%). Volume of systems) 1.85 ml; pH 7.5; incubation temperature 19°. 1) without carnosine; 2) with carnosine.

rate to that found in glycine-buffered systems. Since, in the latter systems, no inactivation of dehydrogenase had taken place, addition of carnosine could have no effect on the reaction.



Composition of systems: fructose diphosphate) 27 μ moles in 1 ml, aldolase) 0.19 mg per ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 18 μ moles in 1 ml, KH_2PO_4) 0.03 M, lactic dehydrogenase) 0.21 mg per ml, PGA dehydrogenase) 0.054 mg per ml, carnosine) 0.022 M (500 mg-%). 0.06 M Glycine or 0.06 M veronal buffers. Volume of systems) 1.85 ml; pH 7.6; incubation temperature 19°. 1) without carnosine; 2) with carnosine.

Fig. 6. Effect of carnosine on the reaction of oxidation of phosphoglyceraldehyde in veronal and glycine buffers.

We found that veronal buffer, like phosphate buffer, inhibited PGA dehydrogenase, and that carnosine also abolished its inhibitory effect (Fig. 6). As with phosphate buffer, the protective effect of carnosine was only

manifested when the PGA dehydrogenase concentration was below certain limits, and consisted in restoring the reaction rate to that found with glycine buffer.

Having established that PGA dehydrogenase is inactivated by 0.12 M phosphate buffer, we proceeded to investigate the relationship between concentration of phosphate and its inhibitory effect on PGA dehydrogenase. Since phosphate is one of the substrates of the reaction, its lack may be a rate limiting factor in the formation reaction of 1,3-diphosphoglyceric acid. On the other hand, excess of phosphate may inhibit the action of the dehydrogenase.

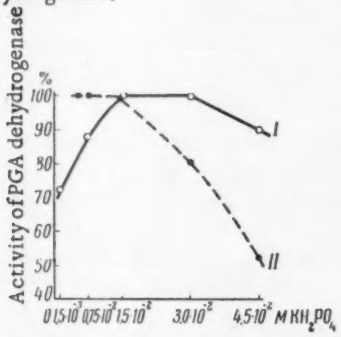


Fig. 7. Dependence of PGA dehydrogenase activity on phosphate concentration. Composition of systems: triose phosphates) 7.0 μ moles in 1 ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 36 μ moles in 1 ml, $NaHCO_3$) 0.05 M, KH_2PO_4) various amounts, lactic dehydrogenase) 0.1 mg per ml, PGA dehydrogenase) 0.05 mg per ml (Curve I) or 0.008 mg per ml (Curve II). Volume of systems) 1.85 ml; pH 8.2; incubation temperature and time) 4 and 8 min at 18°. The progress of the reaction was followed from the rate of increase in 1,3-diphosphoglyceric acid content.

We were able, from the results of a series of experiments involving the use of bicarbonate buffer solutions containing different amounts of phosphate, to find the optimum phosphate concentration for production of 1,3-diphosphoglyceric acid from PGA. The reaction rate was lowered, when the phosphate concentration fell below this optimum value, owing to lack of phosphate for the phosphorolysis of the thio-ester (see Formulation); while at higher concentrations of phosphate, inhibition of PGA dehydrogenase activity was seen. The optimum phosphate concentration varied somewhat with different concentrations of dehydrogenase in the systems. Thus, with a dehydrogenase content of 0.09 mg, maximum reaction velocity was found in systems having phosphate concentrations of 0.015 - 0.03 M (Fig. 7, Curve I). At lower concentrations of enzyme, the optimum phosphate concentrations also became lower (Fig. 7, Curve II).

It would, in accordance with our hypothesis that the effect of carnosine may be ascribed to protection of PGA dehydrogenase from inhibition by phosphate in high concentrations, be expected that at optimum phosphate concentrations, where there is no inhibitory effect, the effect of adding carnosine should also be absent.

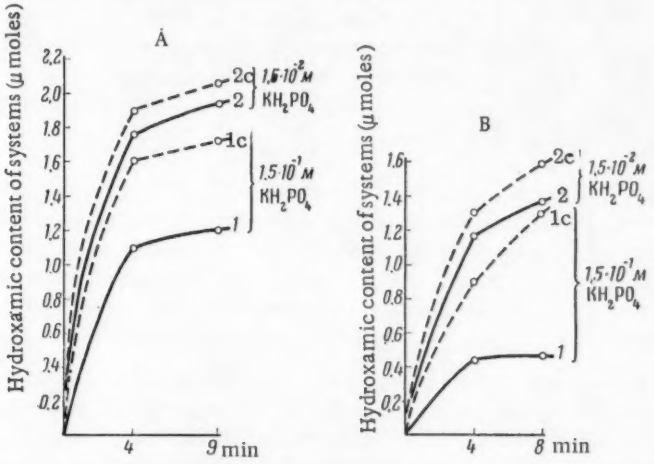


Fig. 8. Effect of carnosine on the oxidation reaction of phosphoglyceraldehyde, at various concentrations of phosphate. Composition of systems: triose phosphates) 7.4 μ moles in 1 ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 36 μ moles in 1 ml, $NaHCO_3$) 0.05 M, KH_2PO_4) 0.015 M or 0.15 M, carnosine) 0.0044 M (100 mg-%), lactic dehydrogenase) 0.1 mg per ml, PGA dehydrogenase) A 0.05 mg per ml, B 0.016 mg per ml. Volume of systems) 1.85 ml; pH 8.4; incubation temperature 19°. 1 and 2) without carnosine; 1c and 2c with carnosine.

As appears from Fig. 8, this expectation was realized. A comparison of Curves I and II (Fig. 8, A) shows that at phosphate concentrations ten times higher than the optimum PGA activity is reduced by 40%. Addition of carnosine almost completely restores the activity of PGA dehydrogenase, whereas it shows very little effect on systems of optimum phosphate concentration (Curves 2 and 2c, Fig. 8, A). The same effect was found for systems of a lower PGA dehydrogenase content (Fig. 8, B). In these systems, phosphate inhibition is more pronounced; addition of carnosine raises the reaction rates almost to the level found under optimum conditions.

From our experimental results we have drawn the conclusion, that the nature of the buffer and the enzyme content of the systems are of importance if it is desired to manifest the stimulatory effect of carnosine on the reaction catalyzed by PGA dehydrogenase. When we incubated systems of low PGA dehydrogenase content (0.016 mg per ml) in 0.15 M phosphate we were regularly able to find inhibition of enzyme activity, which could be fully restored by addition of carnosine.

The action of carnosine is not, however, a specific one. Anserine gave the same effect as carnosine, when added in equimolar amounts. We found that a number of amino acids were able to protect PGA dehydrogenase from inactivation by phosphate, although their effectiveness varied within broad limits. Histidine, taken at the same molar concentrations as carnosine, exerted a more powerful protective action; α-alanine and β-alanine, on the other hand, were only very slightly active in this respect (Fig. 9). Glycine occupied an intermediate position as regards protective activity; its effect was greater than that of equimolar concentrations of α- and β-alanine, but was less than that of carnosine (and anserine). However, as is evident from Fig. 9, the protective effect of glycine could be raised by raising its concentration. Apparently the differences between the protective powers of different aminoacids (and dipeptides) against phosphate inactivation of PGA dehydrogenase bear some relation to their affinity for the enzyme. Of the compounds examined by us, the highest protective power was found for histidine. It might be supposed that the protective power of carnosine is related to the histidine moiety of its molecule, rather than to the β-alanine one.

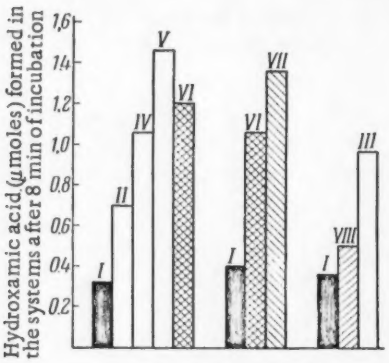


Fig. 9. Effect of carnosine and of some amino acids on the reaction of oxidation of phosphoglyceraldehyde in 0.15 M phosphate buffer. I) without additives; II) 0.0044 M glycine; III) 0.0088 M glycine; IV) 0.0132 M glycine; V) 0.0264 M glycine; VI) 0.0044 M carnosine; VII) 0.0044 M histidine; VIII) 0.0088 M β-alanine. Composition of systems: triose phosphates) 7.4 μmoles in 1 ml, DPN) 0.06 μ moles in 1 ml, Na pyruvate) 36 μ moles in 1 ml, $NaHCO_3$) 0.05 M, KH_2PO_4) 0.15 M, lactic dehydrogenase) 0.1 mg per ml, PGA dehydrogenase) 0.016 mg per ml. Volume of systems) 1.85 ml; pH 8.2; incubation temperature 20°. The time course of the reaction was followed; the histogram shows the amounts of hydroxamic acid formed after 8 min of incubation.

It would be necessary, in order to elucidate the mechanism of this protective action, to know how the inhibiting agent (phosphate, veronal) reacts with PGA dehydrogenase. There are references in the literature which state that veronal [12] and arsenate in high concentrations [13] lower the activity of PGA dehydrogenase. The authors incline to the view that the inhibition is a result of competition for the active centers of the enzyme between substrate molecules (phosphoglyceraldehyde) and veronal or arsenate molecules. Analogously, we may suppose that similar competitive inhibition takes place in the presence of high concentrations of phosphate.

The action of carnosine appears to be the protection of the active centers of PGA dehydrogenase, and the promotion of interaction between the enzyme and its substrate. It is possible that this involves formation of an unstable compound of carnosine with the enzyme. Our attempts at domonstrating the formation of such a compound, using a spectrophotometric method, were unsuccessful; we could perceive no changes in the ultraviolet region of the spectrum of PGA dehydrogenase after incubation with carnosine.

We also examined the effect of pre-incubation of PGA dehydrogenase with carnosine to see whether this treatment made the enzyme more resistant to phosphate inactivation. Our experiments showed that preliminary incubation of the dehydrogenase with carnosine had no effect on its subsequent activity in phosphate buffer. It was only the terminal concentration of carnosine in the reaction mixture which was of significance. The pro-

tective effect was manifested during the course of the reaction only when the enzyme protein, the phosphate, and carnosine or aminoacids were present in certain fixed proportions.

Our finding that high concentrations of phosphate inactivate PGA dehydrogenase does not apply to this enzyme only. A number of enzymes have been shown to be inactivated similarly by phosphate: the activity of urease [14] has been shown to be several times greater in glycine than in phosphate buffer, and succinic dehydrogenase [15] and cytochrome oxidase [16] are markedly inhibited by high concentrations of phosphate. The same applies to glucose-6-phosphate dehydrogenase [17].

The protective action of aminoacids on enzymes has also been noted in a number of cases, and appears to be of fairly general incidence. In particular, the protective action of cysteine on PGA dehydrogenase is well known [18], and may be ascribed to protection of the enzyme from inactivation by pyrophosphate, in the absence of which the effect of adding cysteine is negligible [8]. The use of glycine or histidine buffers [19] provides the most favorable conditions for the activity of mitochondrial enzymes. It is conceivable that under the conditions prevailing in the living cell, in which the concentration of amino acids is, in general, very low, the function of such substances as carnosine and anserine may be to affect the activity of certain of the enzymes, by protecting them from the action of metabolic products arising during the normal functioning of the cell.

The author wishes, in conclusion, to express her profound gratitude to Prof. S. E. Severin for supervising this research.

Received August 15, 1957

SUMMARY

10^{-1}M phosphate inhibits the phosphoglyceraldehyde dehydrogenase. The inhibitory effect depends on the phosphate: enzyme ratio. The addition of carnosine to the reaction mixture neutralizes the inhibitory effect of the phosphate. Anserine as well as certain amino acids exhibit a similar protective action. The compounds tested may be arranged in the following activity series: histidine, carnosine (anserine), glycine, β-alanine (α-alanine).

The second stage of the reaction, viz., transfer to the phosphate residue from 1,3-diphosphoglyceric acid to ADP, is not affected by carnosine. Thus, the stimulating action of carnosine on the phosphorylation-coupled glycolytic oxidation-reduction reaction as noted upon incubation in a phosphate buffer may be accounted for by the neutralization of the inhibitory effect of phosphate upon the phosphoglyceraldehyde dehydrogenase.

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EFFECT OF PHAGE ON THE CHEMICAL COMPOSITION OF BRUCELLA ORGANISMS

I. I. Dubrovskaia, N. N. Ostrovskaia and A. I. Glubokina

Department of Biochemistry and Brucellosis Laboratory, Institute of Epidemiology and Microbiology, Academy of Medical Sciences, USSR, Moscow

The attention of research workers has of recent years been directed to the study of changes in the metabolism of micro-organisms attacked by phage.

Phage, which frequently induces morphological changes in the cells, profoundly disturbs their vital biochemical processes.

Bacteria attacked by virulent phages lose their power of multiplication, of synthesis of adaptive enzymes, and of synthesis of ribonucleic acid (RNA). On the other hand, their desoxyribonucleic acid (DNA) content rises considerably [1].

When infected with phages of moderate virulence, the host cells continue to divide, and retain their power of elaborating adaptive enzymes. One effect, however, is the same for both types of phage infection, viz., increased production of desoxyribonucleic acid, after a transient phase of arrested DNA synthesis [2].

We thought it would be of interest to examine the chemical composition of Brucella organisms after infection with phage, as compared with that of the uninfected organisms.

Our experimental material was a virulent culture of Brucella abortus 146, and Variant 4 of this culture, obtained as a result of exposure of this culture for several hours to an abortus type of bacteriophage.

This variant showed marked morphological differences from the original culture. When plated on agar, the latter gave uniform, convex colonies, with a smooth surface, whereas those of Variant 4 were of different sizes, often with dark inclusions, and the cells were enclosed in mucoid capsules. The phage-infected Brucellae had largely lost their virulence. Whereas the original culture caused generalized brucellosis of mice after inoculation of 10 microbial bodies, 100 million cells of Variant 4 were needed to achieve this effect.

The first few generations of the modified culture were no longer agglutinated by specific serum.

A two-day culture of Variant 4 was plated on liver agar, and incubated for 3 days. The organisms were then washed off with physiological saline, the suspension was centrifuged, and the deposit of microbial bodies was washed three times on the centrifuge with fresh portions of saline. The material was then defatted by suspension in acetone solutions of increasing concentration, and finally in ether, after which it was dried in a vacuum desiccator. The original Brucella abortus 146 strain was treated similarly.

The lipids were recovered from the acetone and ether extracts of the two strains by driving off the solvents in a stream of CO_2. The residues so obtained were dried to constant weight in a desiccator, dissolved in chloroform, and separated by precipitation with acetone into a neutral fat and steroid fraction and a phosphatide fraction.

The yields of lipids and of their fractions from the original and the phage-modified cultures (in %) were as follows:

Microbial material	Lipid content, as % of dry weight of bacterial cells	Neutral fats and steroids	Phosphatides
		as % of total lipids	
Br. abortus 146	9.77	74.80	22.52
Variant 4	12.35	83.29	16.71

If we take the lipid content of the original culture as 100, the lipid content of the phage-infected organisms is 26.4% higher. At the same time, there is a shift in the relative proportions of the two fractions; there is a relative increase in the neutral fat and steroid fraction, and a fall in the phosphatide fraction of the infected organisms.

Since the phage-modified Brucellae possessed a mucoid capsule, the constituents of which might well have passed into solution during the harvesting and washing of the organisms, we also examined the physiological saline used for these operations. After ensuring that it was quite free of microbial bodies, we added two volumes of acetone. The precipitate was collected on the centrifuge, and dried by treatment successively with acetone and ether, and then in a vacuum desiccator (acetone-precipitated fraction).

TABLE 1

Chemical Composition of Br. abortus 146 Organisms, Normal and Phage-Modified

As percentages of dry matter

Material	Reducing subs.	Nitrogen	Phosphorus	RNA	DNA	Hexosamine
Br. abortus 146 Microbial mass	9.82	12.20	1.82	9.22	3.82	3.40
Variant 4 Microbial mass	9.27	12.16	2.24	6.58	7.14	3.05
Acetone-precipitated fraction of wash liquors	26.87	6.51	1.13	4.88	0.51	4.06
Fraction of wash-liquors not precipitated by acetone	18.47	10.91	0.27	2.35	0.0	7.14

The supernatant fluid was freed of inorganic and low-molecular-weight organic compounds, by evaporating off the acetone and dialyzing the residue, and was then freeze-dried (fraction of wash-liquors not precipitated by acetone).

The yields, relative to the microbial mass, were: acetone-precipitated fraction 50 — 55%, not precipitated by acetone 101 -- 117%.

We determined total nitrogen (micro-Kjeldahl), phosphorus (Fiske-Subbarow), reducing substances (Hagedorn-Jensen), nucleic acids (Schmidt and Thannhauser), and hexosamine (Elson and Morgan), in the defatted microbial mass of the initial and the modified cultures, and in the various fractions of the latter recovered from the wash-liquors.

The results are presented in Table 1.

As compared with the initial culture, the phage-modified organisms showed a certain increase in their phosphorus content, and considerable changes in their nucleic acid contents; the ribonucleic acid content was noticeably reduced, while the desoxyribonucleic acid content was very nearly doubled.

Both of the fractions isolated from the wash-liquors differed from the microbial mass in their chemical composition. They contained 2 to 3 times as much reducing substances, but less nitrogen, phosphorus, and nucleic acids.

In the fraction of wash-liquors not precipitated by acetone all of the phosphorus was contained in RNA. This fraction was also distinguished by its high hexosamine content.

We made a qualitative study of the polysaccharide and protein components of the original and the phage-modified Brucella cultures, and also of the fractions isolated from the wash-liquors, by paper chromatography methods.

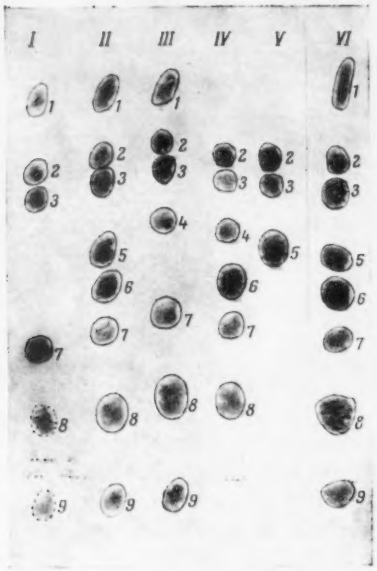


Fig. 1. Carbohydrate composition of microbial bodies of Br. abortus 146, of Variant 4 of this culture, and of fractions of the wash-liquors from Variant 4. I) Original culture; II) phage-modified culture; III) fraction of wash-liquors not precipitated by acetone; IV) and V) control spots; VI) acetone-precipitated fraction of wash-liquors; 1) uronic acids; 2) galactose; 3) glucose; 4) mannose; 5) arabinose; 6) xylose; 7) ribose; 8) rhamnose; 9) fast-running component.

Hydrolyzates of the polysaccharides were prepared by the action of N H_2SO_4 for 5 hr , on the microbial bodies and the fractions derived from them. After separating the hydrolyzates from insoluble matter, and after elimination of excess sulfuric acid, we treated them with chloroform, according to Sevag [3], in order to remove proteins, and then with alcohol, and concentrated the solutions down to specified volume. The chromatograms were spotted with amounts of hydrolyzate having a reducing sugar content equivalent to 150 μg of glucose.

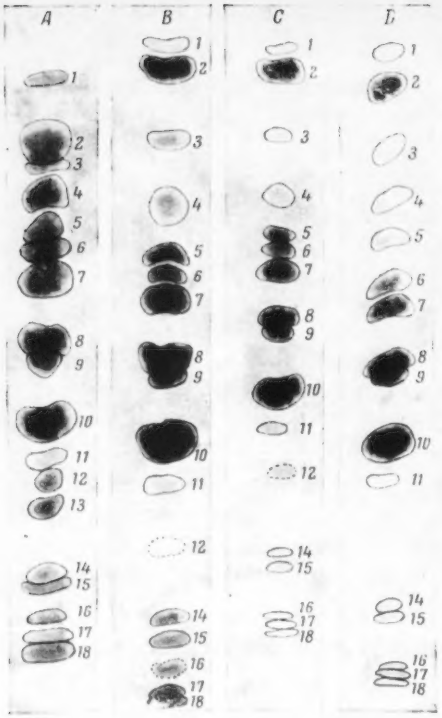
The paper was of a parchment base type, and the solvent system used consisted of butanol – acetic acid – water (40 : 10 : 50); aniline phthalate was used as the spray reagent.

A photograph of the chromatograms is shown in Fig. 1.

It is evident from the chromatograms that the composition of the bacterial polysaccharides is changed following phage infection. In addition to uronic acids, galactose, glucose, ribose, rhamnose, and a fast-running component [4] found in the original strain of Br. abortus 146 (I), the polysaccharides of the phage-modified culture (II) also contained arabinose and xylose. Judging from the intensity of coloring of the spots developed from amounts of hydrolyzate of the same reducing sugar content, there is an increase in the rhamnose and fast-running component contents of phage-modified organisms.

The fraction precipitated by acetone from the wash-liquors does not differ in carbohydrate composition from the product obtained from phage-modified microbial bodies (VI). The composition of the polysaccharides of the fraction not precipitated by acetone (III) is, however, quite different. Arabinose and xylose are absent, but a new hexose – mannose – is found. The presence of mannose was confirmed by formation of phenylhydrazone crystals in the cold. It is possible that these polysaccharides enter into the composition of the capsules forming around phage-modified Brucellae, and represent capsular polysaccharide, differing from the somatic polysaccharide of the microbial bodies.

In order to investigate the amino acid composition of the proteins of Brucella cells and of the derived fractions we subjected them to hydrolysis with 6 N HCl for 24 hr. The hydrolyzates were freed of humin substances and of excess acid, and evaporated to dryness, and the residue of amino acids was extracted with acidified acetone [5], in order to separate them from salts. The acetone was cautiously evaporated off. The residue was dissolved in the minimum amount of water, and the solution was taken for chromatography, applying a volume containing 30 μg of protein N. The paper used was "Leningrad Rapid," the solvent system was butanol – acetic acid – water (40 : 10 : 50 and 40 : 15 : 5), and the spray reagent was ninhydrin. For the better separation of the amino acids we ran the solvent down the chromatograms three or four times, after drying the paper.



A) Microbial mass of original culture; B) microbial mass of phage-modified culture (Variant 4); C) acetone-precipitated fraction of wash-liquors; D) fraction of wash-liquors not precipitated by acetone. 1) Cystine; 2) lysine; 3) histidine; 4) arginine; 5) aspartic acid; 6) serine; 7) glycine; 8) glutamic acid; 9) threonine; 10) alanine; 11) proline; 12) tyrosine; 13) α-aminobutyric acid; 14) methionine; 15) valine; 16) phenylalanine; 17) leucine; 18) isoleucine.

Fig. 2. Amino acid composition of proteins of microbial bodies of Br. abortus 146, of Variant 4 of this strain, and of fractions isolated from the wash-liquors of cultures of Variant 4.

Photographs of the chromatograms are shown in Fig. 2.

The proteins of the microbial bodies of the original culture of Brucella contained 18 amino acids: cystine, lysine, histidine, arginine, aspartic acid, serine, glycine, glutamic acid, threonine, alanine, proline, tyrosine, α-aminobutyric acid, methionine, valine, phenylalanine, leucine, and isoleucine. In addition, spraying with isatin revealed hydroxyproline.

The same amino acids were found as components of the proteins of phage-modified organisms, with the exception of α-aminobutyric acid. This amino acid could not be detected even when a triple amount of hydrolyzate (90 μg N) was taken. Judging from the depth of coloring of the spots, the proteins of the phage-modified cultures were richer in lysine and alanine, but poorer in tyrosine and phenylalanine, than were those of the original culture.

The proteins of the wash-liquor fractions had the same amino acid composition as those of the phage-modified microbial bodies. They likewise did not contain α-aminobutyric acid, and had a low tyrosine content.

The proteins of the fraction not precipitated by acetone did not give a tyrosine spot when an amount of hydrolyzate containing 30 μg of protein N was applied. A very faint spot appeared when the amount of hydrolyzate was doubled. The proteins of this fraction were also distinguished by their very low contents of methionine, valine, phenylalanine, and leucine.

It might be supposed, from the polysaccharide and amino acid compositions of the fraction of wash-liquor precipitated by acetone, that this fraction consists chiefly of lysed microbial bodies. The fraction remaining in solution in 66% acetone is probably a component of the mucoid capsule formed around phage-infected organisms.

Our results show that infection with phage causes a profound disturbance of the metabolism of Brucellae, leading to marked changes in the chemical composition of the organisms.

SUMMARY

Phage-infected cultures of Brucella abortus 146 are distinguished by a raised lipid content of the organisms, chiefly due to increase in the neutral fat and steroid fraction, a considerable rise in the desoxyribonucleic acid content, and a fall in the ribonucleic acid content.

The polysaccharides of the organisms of the phage-modified cultures contain, in addition to uronic acids, galactose, glucose, ribose, rhamnose, and a fast-running component, also found in the original culture, two pentoses, arabinose and xylose.

The hydrolyzate of the original organisms was shown chromatographically to contain 18 amino acids, including α-aminobutyric acid. This amino acid was not found in hydrolyzates of the phage-modified cultures.

The physiological solution used for washing the phage-modified organisms contained a fraction, not precipitated by adding acetone to 66%, which differed in composition from the microbial bodies; it did not contain desoxyribonucleic acid, and its specific polysaccharides did not contain arabinose or xylose, but contained mannose. This fraction probably originates from the mucoid capsule found in phage-infected Brucellae.

Received July 16, 1957

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ON CERTAIN ENZYMES OF PHOSPHORUS METABOLISM AND THE INTENSITY OF RESPIRATION AND AEROBIC GLYCOLYSIS IN THE DEVELOPING STURGEON EMBRYO

R. I. Tatarskaia, K. K. Kafiani and S. I. Kanopkaite

Department of Animal Cellular Biochemistry, The A. N. Bakh Institute of Biochemistry, USSR Academy of Science, Moscow

It was shown previously that the levels of the main phosphorus compounds altered very little during the development of sturgeon eggs. However, when eggs from sturgeon of a related species were allowed to develop in the presence of P^{32}-labelled phosphate, the isotope was found to be rapidly incorporated into the various fractions [1].

It seemed of some interest to investigate the rate of change in activity of certain enzymes concerned with phosphorus metabolism during the embryonal development of the sturgeon eggs. The enzymatic activities investigated were acid and alkaline phosphatases, apyrase and the intensity of enzymatic hydrolysis of various endogenous substrates, mainly phosphoproteins, at various pH values. The investigation also included assays of intensity of both respiration and aerobic glycolysis, since both these processes lead to the formation of energy-rich phosphorus compounds. The choice of enzymes investigated was partially affected by the fact that most of the investigations were carried out under field conditions where research facilities were somewhat limited.

There is in existence a rather copious literature dealing with the alterations in enzyme distribution and their significance during the embryonal development of various animals; there are also in evidence many disparities between existing data. Histochemical evidence points to the predominance of alkaline phosphatase in tissues concerned with intensive protein synthesis – mammary glands, regenerating tissue, hair roots, etc. Phosphatase reaction in the embryonal tissues is faint in the initial stages, but becomes more pronounced with the onset of differentiation and the concomitant intensification of protein synthesis [2]. In some organs this enzyme can only be demonstrated during embryonal development. Unfortunately, it does not seem possible to make use of all the existing histochemical data dealing with the distribution and localization of alkaline phosphatase in tissues, since critical reappraisal of modern histochemical methods showed them to be unreliable in the study of cellular structures [3,4]. The study of phosphatase distribution in various cell components isolated by differential centrifugation showed that in liver, both the acid and alkaline phosphatase were localized in the cytoplasm, the former being associated mainly with the cytoplasmic granules; and the latter, with the cell sap (supernate) [3,5].

Acid phosphatases have been found in many tissues of adult animals as well as in some developing eggs. The involvement of this enzyme in nucleic acid synthesis has also been observed in some instances [6]. However, in distinction from alkaline phosphatase, the character of the change of acid phosphatase activity in the course of embryonal development differs in the various species.

A large number of investigations deal also with the various enzymes which split off high-energy phosphate groups from adenosinetriphosphate (ATP). Investigations into the nature of muscle ATP-ase demonstrated the identity of this enzyme with the contractile muscle protein – myosin [7].

The chemical energy of ATP is in constant demand during the embryonal development for the synthesis of proteins and other cell ingredients. In accordance with terminology previously employed in this laboratory [8],

ATP breakdown is thought to be better expressed by the term adenosine polyphosphatase (APP-ase), determined as total phosphate split from ATP.

In the developing embryo ATP-ase activity of various organs increases quite sharply, the rate of its increase considerably exceeding that of phosphomonoesterase activity [9,10]. It should be kept in mind that toward the end of the developmental period the distribution of various phosphates in the organs will vary depending on their future function. Thus, in the chick embryo, alkaline phosphatase activity in the gut and ATP-ase activity in the muscle increase sharply immediately before hatching [10].

In view of the presence in various animal eggs of varying amounts of yolk mainly composed of phosphoprotein (PP), there is a considerable volume of literature dealing with the enzymes concerned with the metabolism of these substrates. The data, however, are not in good agreement. In the first instance, rather diverse data are quoted for the degree of yolk utilization during embryonal development even in the case of closely related or identical species, when employed by different authors [11,12]. According to our observations [1], the PP content of sturgeon eggs remained practically unaltered up to the hatching stage, a fact reported also by Barth and Barth [12] when working with the eggs of tailless amphibians. The enzyme PP-phosphatase was originally observed in ova of Rana pipiens and R. catesbiana by Harris [13]. The intracellular localization of this enzyme varied, depending on the type of substrate employed (casein, actual egg PP). The majority of authors, however, consider it to be localized within the yolk [12,14]. There is also little agreement about the properties of this enzyme. According to some authors the pH optimum for its activity ranges from 3.8 to 7. There is also disagreement with regard to activating and inhibitory agents. The conditions under which PP-phosphatase activity was determined by various authors varied; incubation times alone ranging from 14-30 min to 10 hours and longer [12-14]. The variance of all these data need not be surprising in view of the indications by Perlman as well as by Hofman [15], that PP-phosphatase is not a single enzyme but is probably a mixture of a number of phosphatases. Nass [14] has also demonstrated that, all other conditions being equal, the amount of mineral phosphate split from endogenous substrate in short term incubations depended on the degree of solubilization of the yolk employed. It would appear that during the utilization of various substrates (casein, chick egg vitellin, endogenous substrates of the amphibian egg), different phosphate bonds are hydrolyzed, which presumably involves the activity of different enzymes. For that reason all reference in the present communication to PP-phosphatase activity will mean the ability of egg homogenates to split inorganic P from natural substrates at pH 5; it will be shown further that the hydrolysis of natural PP by egg homogenates takes place only in acid medium.

The problem of egg respiration in the ontogenesis of sturgeon has also been dealt with in a number of communications, the results obtained lacking agreement with each other. Thus, there are reports about the gradual fall in respiratory activity of sturgeon eggs during embryonal development [16], about alternating periods of elevated and depressed respiration rates (the reported number of such periods varying from author to author and sometimes in the hands of one author [17]); a gradual increase in respiration rate during egg development has also been reported [18].

In view of the fact that the majority of present investigators dealing with the respiration during ontogenesis agree that the intensity of this process increases throughout the development of the egg in accordance with its increased content of "active" plasma (cf., for instance, [19]), it was of interest to explain the reason for the existing diversity of data about the respiration rates of developing sturgeon eggs and to discover a possible pattern for the variation of this process. It was also thought useful to measure the intensity of the aerobic glycolysis during the various periods of egg development. It has been claimed in the communications cited above that the periods of elevated respiratory activity were characterized by a decrease in glycolytic rates and, inversely, that an increase in glycolysis was observed during the periods of lowered respiration [17, 20, 21].

EXPERIMENTAL

Materials and Methods

Developing eggs of two species of sturgeon, sevriuga and osetr,* were used in the experiments. Because of the short spawning period of the sturgeon, and the lack of suitable laboratory facilities on the piscicultural station at Rogozhno on the Don estuary, where the material was procured, only part of the work was carried out

*Russian sturgeon, Acipenser guldenstadti.

on living eggs. Apart from the living eggs, frozen material as well as freeze-dried material was also used. It was established in control experiments that the general pattern of activity was unaltered in the fixed material, although the absolute levels of activity were somewhat lower than in fresh eggs. The determination of optimal pH for enzymatic activity was carried out with a buffer series. The various buffers used were glycine, acetate, borate, veronal,and carbonate, within the pH range 1.42 to 10.46, determinations being made at 0.5-0.7 pH unit intervals.

It will be seen from the graph (Fig. 1) that during stage 30 in the development of the sturgeon egg, the phosphomonoesterase activity of the homogenate has two pH optima – at pH about 4.8 and about 9, respectively, corresponding to the acid and alkaline phosphatases (in the present instance and throughout this communication the nomenclature of the developmental stages will be according to Detlaf and Ginzburg [22]). It should be noted that the maximal splitting of endogenous egg substrates also occurred in the same pH ranges. Identical results were obtained with both species of sturgeon used. The minimal phosphomonoesterase activity was observed in the pH region 5.5-7.5, in which the APP-ase activity was found to be maximal (Fig. 2). ATP-ase showed a flat pH optimum in the range 6.6-7.5, and this circumstance permitted determination of APP-ase activity with good accuracy under conditions where phosphatases were almost inoperative. Any possible error in APP-ase assay due to phosphatase activity was, in any case, negligible because of the comparatively high APP-ase activity.

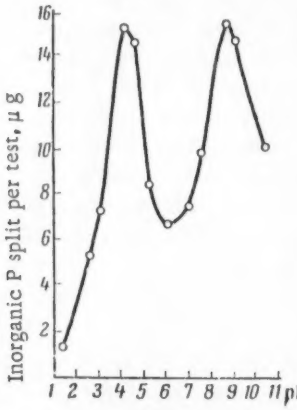

Fig. 1. The effect of pH on the total mineralization of phosphorus from endogenous substrate and from β-glycerophosphate in osetr egg homogenates (stage 30).

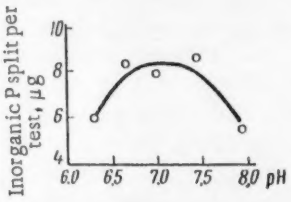

Fig. 2. The effect of pH on the cleavage of inorganic P from ATP by homogenates of sevriuga eggs (stage 30).

The character of the changes in all the enzymatic activities mentioned was followed up throughout the embryonal development of the sturgeon eggs, using homogenates of the developing embryo in all stages, from the non-fertilized mature egg to the newly hatched larva.

In the determination of phosphomonoesterase activity, the eggs (20 or 40 in number) were homogenized in the cold, first with a small amount of distilled water, and then with the subsequent addition of 4 or 6 ml of the appropriate buffer (the amount of buffer added comprised 3/4 of the final volume). Determinations were carried out using 1 ml of the homogenate and 1 ml of a solution containing 0.7 mg of calcium β-glycerophosphate (about 4 μ moles) and $MgCl_2$ in the concentration 1×10^{-3} M. Control determinations were carried out with $MgCl_2$ alone. The Ca^{++} ions, in the concentration employed, did not affect the amounts of inorganic phosphate in control determinations in both the acid and alkaline medium. The reaction was stopped after a one-hour incubation at 37° by the addition of 0.9 ml of 10% trichloroacetic acid (TCA). It was found in control experiments that the glycerophosphate preparation employed contained no inorganic phosphate, nor was the latter liberated when the enzyme was left out of the incubation mixture.

APP-ase activity was assayed by a slightly modified procedure of Gustafson and Hasselberg [23]. Several samples (each containing 20 eggs) were homogenized in cold 0.7% KCl. The homogenate was then diluted with 0.9% KCl to 2 ml, followed by the addition of 0.1 ml of 0.04 M $CaCl_2$ and 1.9 ml of M/20 borate buffer pH 7.5. The tubes were placed on the water bath at 23°, allowed to equilibrate, and the reaction was started by the addition of 1 ml of 0.2% ATP solution (2 mg), previously adjusted to pH 7.5. Incubation was continued for 15

min and the reaction was stopped by the addition of 4 ml of 15% TCA (control tubes received ATP immediately before the addition of TCA). Blank determinations of non-enzymatic ATP cleavage under incubation conditions were also carried out.

PP-phosphatase activity was assayed as inorganic P liberation in reaction mixtures incubated in the presence of acetate buffer, pH 4.8, under conditions similar to those employed in acid phosphatase assay, but without the addition of glycerophosphate. Special experiments, to be dealt with below, showed that only at the pH value mentioned was it possible to observe a partial decomposition of PP. It appears that endogenous substrates of some other nature are cleaved at pH 9. Deviations from the PP-phosphatase assay method described here, will be dealt with in detail in the text.

Respiration rates were determined on the eggs of both sturgeon species employed at all the stages where enzymatic activities were assayed. The determinations were made gasometrically in a Barcroft apparatus. As is well known, the sensitivity of this apparatus is somewhat below that of Warburg, nevertheless it has an invaluable advantage for field work in that its readings are practically independent of temperature variations in the surrounding medium [24], while in the Warburg apparatus variations in bath temperature of the order of several hundredths of one degree may introduce considerable errors into the results.

Vessels of about 20 ml capacity received 40-80 osetr eggs or 60-100 sevriuga eggs, and the total volume was adjusted with water to a value which was the same for all vessels. The gaseous spaces in paired vessels of each apparatus were equal. Incubations were carried out for 60-75 min at room temperature (20-25°), care being taken to ensure that each batch of eggs was incubated at an even temperature (variations not exceeding 0.2-0.3°) throughout the period when respiration rate measurements were taken. The apparatus was carefully shaken periodically (40-50 times a minute). The liberated carbon dioxide was absorbed by concentrated potassium hydroxide placed in the central well. Readings were taken every 15 min.

Statistical analysis of results showed that the value of the standard deviation varied from ± 1.98 to ±6.86%, and the standard error of the mean in the various experiments ranged between ±1 and ±3%.

At the end of the determinations the eggs were usually left in for several hours in the apparatus and their development was compared with that of control batches. No deviation from the normal development could be detected in eggs employed in the respiratory studies. There are also indications in the literature that cautious shaking does not interfere with developing eggs. Thus, in the experiments of Ten Cate [25], developing frog's eggs were also shaken in the Warburg apparatus; at the very early developmental stages, when the embryos (devoid of the gelatinous membranes in the particular experiments) showed some sensitivity, the shaking frequency was merely decreased (to 10-15 per minute). Sturgeon embryos, rather similar to those of frogs , developing generally under natural conditions involving rapid streams, were found to be quite resistant to gentle shaking.

In order to determine the nature of the enzymes responsible for the increase in respiration rates during embryonal development, certain determinations were carried out in the presence of varying concentrations of potassium cyanide. Details of these experiments will be dealt with in the appropriate place.

Aerobic glycolysis rates were also determined with sevriuga eggs. For this purpose aliquots of eggs (100 eggs each) were fixed with boiling alcohol at various stages of development. The alcohol was then distilled off in vacuo and the residue extracted with 10% TCA. The fixed eggs were homogenized and extracted several times with 10% TCA. All acid extracts of each aliquot were combined and the lactic acid content of the pooled extract was determined according to Barker and Summerson [27].

RESULTS

Alkaline Phosphatase

Sturgeon eggs in the initial developmental stages showed very slight activity in splitting glycerophosphate at pH 9. Glycerophosphatase activity could not be detected at all in some batches of unfertilized eggs and in eggs undergoing the first stages of division.

The change in alkaline glycerophosphatase activity in one of the batches of eggs employed is shown in Fig. 3. (sevriuga eggs, fresh material). The activity was fairly low until stage 26-27, after which the splitting of glycerophosphate became intensified, reaching activity levels of 16 μg P per 10 eggs per hour at hatching

stage. A final activity of the same order was also observed in other batches of eggs.

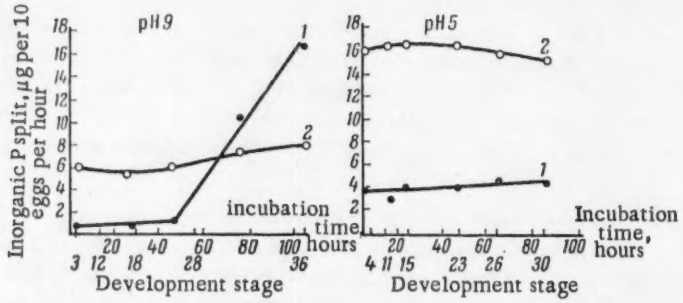

Fig. 3. The change in alkaline and acid phosphatase activities during the development of sevriuga eggs: 1) Mineral P splitting from added β-glycerophosphate; 2) Mineral P splitting from endogenous substrate.

Although the splitting of endogenous substrate at pH 9 was somewhat higher at the early stages than glycerophosphate splitting, this former activity was still very small compared to the alkaline glycerophosphatase at the late stages and, moreover, it changed little throughout the developmental period.

Acid Phosphatase

A completely different pattern of activity during the embryonal development was found to apply in the case of acid phosphatase (Fig. 3). This enzyme was found to be quite active even in the non-fertilized eggs (in the given batch, about 4 μ g P per 10 eggs per hour). However, throughout the developmental period the acid phosphatase activity remained virtually unaltered. It should be noted that, under the acid conditions, the amount of inorganic P split from endogenous substrate was several times higher than that split under the same conditions from β-glycerophosphate (about 16 μ g per 10 eggs per hour in the given batch of eggs at the same stage). This value corresponds with that of alkaline phosphatase activity at the very latest stages of development. It is also noteworthy that the splitting of endogenous substrate at pH 5 remains at approximately the same level throughout the development of the embryo.

Adenosinepolyphosphatase Activity

Presented in Fig. 4 is a typical curve showing the change in APP-ase activity during the development of sevriuga eggs. This activity was found to be quite high even in the earliest stages, reaching 2 μ g P per one egg in 15-min incubation. APP-ase activity was found to undergo little change up to stage 28, after which it increased rapidly so that, toward the last developmental stages, the rate of P-splitting increased about six-fold.

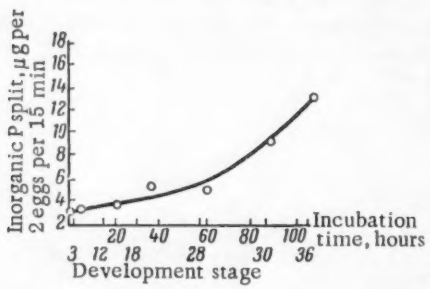

Fig. 4. The change in adenosinepolyphosphatase activity during the development of sevriuga eggs.

Thus, the behavior of APP-ase activity during the development of the embryo was found to be similar to that of alkaline phosphatase. It must, however, be borne in mind that the similarity involved the pattern of change; the absolute activity levels being quite different. Thus, if the data obtained is compared at the final developmental stage, the values being extrapolated to similar conditions of concentration, temperature and incubation time, it is easily seen that the value of alkaline phosphatase activity was only about 1/30 that of APP-ase activity.

As mentioned above, it may be assumed that APP-ase activity increased in connection with the differentiation of muscular tissue and the preparation for motor function. Attempts were therefore made to

isolate the protein fraction corresponding to myosin from the homogenates of developing eggs at various stages, using the accepted method for its isolation [28], and to determine the APP-ase activity of the isolated fractions. The work was carried out under field conditions, without suitable means of cooling the solutions sufficiently, which could not have failed to cause some loss of enzymatic activity; a constant increase in APP-ase activity in the specific fraction isolated was, nevertheless, established quite definitely.

Phosphoprotein Phosphatase Activity

In view of the diversity of existing data in the literature relating to PP-phosphatase activity, it was considered advisable to establish, in the initial experiments, whether or not homogenates of developing sturgeon eggs were at all capable to mineralize the phosphorus of either added or endogenous phosphoprotein.

1. Experiments with casein. The added substrate which was employed in the PP-phosphatase assay was casein which was freshly precipitated from an alkaline solution, washed with distilled water and lyophilized. The preparation contained no inorganic phosphate and had the appearance of a light powder, easily soluble in neutral solutions.

The capacity of homogenates to mineralize casein phosphorus was determined at both pH 4.8 and 9.0, since it has been shown above in the case of endogenous substrates, that very little activity took place at other pH values (Fig. 1). Casein was added as a neutral aqueous solution (600 mg per tube); the casein solution was replaced by water in control incubations. Determinations were also made of the non-enzymatic cleavage of the casein phosphorus under experimental conditions (with negative results), as well as of the initial amounts of phosphate present in the sample of eggs added. The reaction mixtures were stirred continuously for one hour.

It will be seen from Table 1 that the phosphorus was actually split from casein, even though the absolute amount hydrolyzed was not high, and that the extent of hydrolysis at the alkaline and acid pH was approximately the same.

The results allow the conclusion that sevriuga eggs contain either two PP-phosphatases, or one PP-phosphatase with two pH optima.

TABLE 1

The mineralization of casein phosphorus by homogenates of sevriuga eggs at various pH values. In μg P split from casein, per 10 eggs

Development stage	pH	
	4.8	9
30	2.7	2.0
35	2.6	2.4

Note: All tests were carried out in triplicate or quadruplicate.

2. The splitting of endogenous phosphoproteins. Entirely different results were obtained when the same biological material was used for the determination of inorganic P split from endogenous substrate, carried out simultaneously with the measurement of decrease in PP after incubation (PP analysis was according to Schmidt and Thannhauser [29]).

It will be seen from the results presented in Table 2 that the P from endogenous PP was mineralized by the sevriuga egg enzymes only at the acid pH. PP hydrolysis at pH 9 was quite insignificant — the values obtained fell within the range of experimental error.

Similar results were observed with a batch of eggs taken at an earlier developmental stage, but here, as a rule, the amount of inorganic phosphate gained was about one-half to one-third of that of the phosphoprotein P lost. It would appear that concomitant with the splitting of the phosphorus, there also took place partial proteolysis of PP which produced some TCA-soluble protein fragments. Indeed, since the experiments were carried out with homogenates and not with purified enzymes, the participation of proteolytic enzymes in the reaction could not be excluded. The problem requires further study, particularly since the determination of PP-phosphatase activity by the method involving the decrease of PP-phosphorus was not carried out at all the developmental stages, and therefore any generalizations on the behavior of this activity through embryogenesis would be premature.

Another problem arises from the data presented above. Since some unknown endogenous substrates other than PP were cleaved at pH 9, it was not clear what caused the low level of activity of this process — whether it was actually due to low levels of enzymatic activity or whether, perhaps, the accessible substrates were

TABLE 2

The disappearance of endogenous phosphoprotein during the incubation of sevriuga egg homogenates at various pH values

Development stage	Initial PP-phosphorus content	PP-phosphorus content, in µg per 10 eggs after incubation	
		pH 4.8	pH 9
35	154	131	152
	Loss of PP-phosphorus	23	2

Note: All values represent means of four or five determinations.

TABLE 3

The effect of cysteine in various concentrations on the cleavage of phosphoprotein-P in osetr egg homogenates (Stage 17)

Cysteine concentration, M	Increase in inorganic P, in µg per 10 eggs after 120 min incubation	
	expt. 1	expt. 2
0	20.3	13.3
10^{-2}	—	13.7
10^{-3}	21.0	—
10^{-4}	21.1	—

Note: It has been established in control experiments that, in the concentrations employed, cysteine did not interfere with the estimation of inorganic P.

TABLE 4

The effect of addition of magnesium to incubated homogenates of osetr eggs (Stage 17) on the subsequent content of inorganic and total phosphorus and of total nitrogen in the TCA extract

Incubation time: 120 min

additions	inorganic P split	increase in total P	increase in total nitrogen
	µg per 10 eggs		
in the absence of $MgCl_2$	18	9	7.9
in the presence of 10^{-4} M · $MgCl_2$	18.4	13	9.3

limiting. It was therefore impossible to draw any conclusions about the behavior of this activity during the development of the eggs.

3. The effect of added Mg and cysteine on the splitting of mineral P from endogenous substrates, and on the increase in total P and nitrogen in the TCA extract after the incubation of homogenates at pH 4.8. According to Sundararajan and Sarma [13], beef spleen PP-phosphatase activity could be increased 5-6 fold by the addition of compounds containing the –SH group (cysteine, thioglycolic acid). The effect of various concentrations of cysteine on the activity of sturgeon egg PP-phosphatase was therefore investigated (Table 3).

Experiments were also carried out in order to assess the effect of Mg^{++} on the splitting of inorganic P from endogenous substrates and on the increase in total P and nitrogen in the TCA extract. As will be seen from Table 4, the addition of Mg^{++} had no effect on the splitting of inorganic P from endogenous substrate under experimental conditions. The rise in total P in the TCA extract of the incubation mixture was generally small, and increased only slightly in the presence of added Mg^{++}. A slight increase in total nitrogen was also observed. It follows from the results obtained that at the concentrations employed, neither magnesium nor cysteine showed any appreciable activating effect on PP-phosphatase, the latter activity being taken as the ability to split inorganic phosphate from endogenous substrates at pH 5.

The Respiration of Developing Sturgeon Eggs

The graphs presented in Fig. 5 illustrate the changes in respiratory intensity of the developing sturgeon eggs. The data were obtained in several seasons with different batches of eggs. Use was made of eggs incubated in industrial-hatching equipment in the open air, in the months of June and May, in water at varying temperatures (16-23°). It is therefore not surprising that the respiration curves differed from each other. Nevertheless, obvious in all samples was an uneven but continuous increase in respiratory activity throughout the development, which resulted in most of the curves assuming a sigmoid shape. No constant inflexion points could be detected common to all curves. The rate of gain in respiratory activity usually increased sharply before hatching (in the case represented by Curve 4, obtained with a batch of eggs whose development was retarded, the rate of increase was lower than with other batches).

The mean oxygen consumption by sevriuga eggs was found to be, in our hands, 2.5-3.0 µl per 10 eggs per hour in the initial development stages, and 12.5-13 µl per 10 eggs per hour before hatching. The respiration curves obtained with osetr eggs were of similar character, but the oxygen consumption in this case was about 1.5 times that of the former.

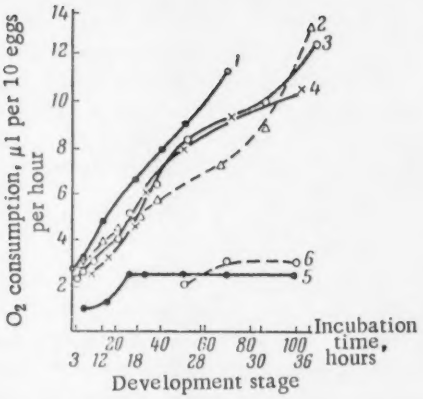

Fig. 5. The changes in respiration intensity during the development of sevriuga eggs. 1,2,3, and 4) normal respiration curves obtained with various industrial batches of roe; 5 and 6) the roe showed in Curve 1, incubated in the presence of 0.003 M and 0.006 M KCN, respectively. Temperature of measurements: 22° (Curves 1,4,5 and 6), 24° (Curve 3) and 25° (Curve 2).

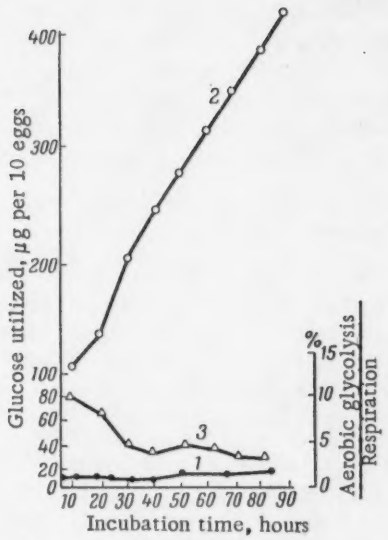

Fig. 6. The comparative intensities of respiration and aerobic glycolysis: 1) aerobic glycolysis (lactic acid content); 2) respiration; 3) per cent ratio of respiration and glycolysis (it was assumed, for the sake of simplicity, that the two processes utilized glucose exclusively, and their respective intensities have been expressed as µg of glucose consumed.

In order to elucidate the effect of cyanide on the respiration of developing sturgeon eggs the inhibitor was used in concentrations $1 \cdot 0$-, 3.0- and 6.0×10^{-3} M. It is obvious from Fig. 5 that no purpose could be served by increasing the cyanide levels further, since the constant level of residual respiration was reached already at a concentration 3.0×10^{-3} M. The absolute level of the residual respiration remained constant from the blastula stage onwards. It therefore follows that the increase in oxygen consumption during the development of sturgeon eggs is due mainly to the increase in cytochrome respiration.

The lactic acid content of sevriuga eggs in the course of their development was found to be comparatively low — from 11 µg per 10 eggs in the initial stages to 16 µg at the time of hatching, with slight variations in the intermediate period. It should, of course, be kept in mind that lactic acid content as determined at any one moment in aerobiosis cannot provide an exact estimate of the intensity of glycolysis, since at that time the processes responsible for lactic acid formation take place simultaneously with those leading to its destruction (the reverse reaction, further oxidation etc.).

It would, however, appear that irrespective of the mechanism responsible for its appearance, the lactic acid content throughout the aerobic development was very low. This tends to point out that in the general energetic balance of the developing egg the energy of glycolysis comprises a rather insignificant proportion. On the assumption that the respiration of the developing eggs was based entirely on glucose oxidation, and using the available mean values for oxygen consumption in the various 12-hour periods (calculated on the basis of available data obtained in the different developmental periods), it was possible to calculate the glucose consumption for each of the consecutive 12-hour periods (Fig. 6, Curve 2). The intensity of glycolysis could be similarly represented (Curve 1), since it is known that the amount by weight of lactic acid formed in glycolysis is equal to that of glucose utilized. If the glycolytic activity is further expressed as per cent of respiratory activity, the pattern obtained is that shown by Curve 3; it is seen from this curve that the onset of development (under aerobic conditions) the contribution of glycolysis to the energetic balance was of the order of 10%. This figure fell to 4-5% toward the beginning of gastrulation. This latter ratio of glycolysis to respiration prevailed to the end of the development, showing but a slight tendency to fall.

DISCUSSION

The investigations showed that most of the enzymes assayed (ATP-ase, acid phosphomonoesterase, PP-phosphatase) showed a definite activity in the earliest stages of development, including the non-fertile, mature egg. Other enzymes (eg.,alkaline phosphatase), which showed only weak activity during the initial stages of development, were greatly activated during its subsequent progress. The increase of activity of alkaline phosphatase and ATP-ase was particularly remarkable toward the end of the developmental period when intensive embryo formation was taking place, accompanied by active tissue growth and organ differentiation.

It seems of some interest to compare the results obtained in the present work with available information relating to phosphatase activity changes during the embryonal development of other animals.

As has been mentioned above, alkaline phosphatase activity increases during the embryonal development of all species, but the period during which this increase takes place varies with the different species. Thus, in the sea-urchin this activity rises after gastrulation [30]; in the chick embryo it increases only after the sixth day of development, which coincides with the onset of energetic organogenesis [9,10]; in amphibians the steep increase in alkaline phosphatase activity is observed, as in the sturgeon, after neurulation, particularly from the stage of tail-bud formation.

No general pattern was observed regarding the behavior of acid phosphatase in the developing ova of various animals. In the chick embryo the acid phosphatase is activated at the same stage as the alkaline phosphatase, the former lagging considerably behind the latter in the rate of increase [10]. In the sea-urchin [23;30] and the amphibians [11] acid phosphatase activity remains virtually unaltered throughout the development. As seen from the present results, this also held for the sturgeon eggs.

Apyrase, or as it has been referred to here, APP-ase, also undergoes a considerable increase in activity during embryogenesis. This increase has already begun in the earliest stages of development in the sea-urchin egg [23,31]; in the chick embryo APP-ase activity shows one increase after the sixth day of development [9], and another during the second half of the incubation period in connection with the functional activity of the tissues [10]; in amphibian eggs APP-ase activity begins its increase after gastrulation [31]. As shown above, in the developing sturgeon egg APP-ase activity became activated after neurulation, and particularly after the appearance of the tail-bud. Thus, in this case also there is considerable similarity in the behavior of amphibian and sturgeon embryos.

As was mentioned above, PP-phosphatase is still not sufficiently well known. A single mention of its behavior during embryogenesis is found in the work of Metzger-Freed [13]. In this work PP-phosphatase was assayed in developing frog eggs by measuring the hydrolysis of endogenous substrates at pH 3.8. The author claimed that variations in PP-phosphatase activity throughout the development period were insignificant.

It may be concluded, on summing up the available information, that the behavior of the enzymes investigated in the developing sturgeon eggs resembled most closely the pattern observed in the tailless amphibians. The strong similarity in external appearance of the eggs of these two groups has already been mentioned [22]. The present results provided additional evidence about their similarity in the biochemical sense.

It can also be stated, with regard to the respiration of sturgeon embryos, that the intensity of their respiration changed during their development in accordance with the general pattern which has been observed with a large number of animal species investigated, including fishes (cf., for instance, [32]). The present results are in disagreement with those of some other authors [16, 17, 20, 21], who investigated the respiration, glycolysis and other biochemical indices of developing fish embryos in connection with the "critical periods" of the embryonal development.

According to the authors referred to above, the critical period of the development of an embryo is understood to be one during which the embryo is particularly sensitive to the action of damaging agents and when the processes of differentiation are at maximum while growth is weakened. The authors also believe that these periods are characterized by the presence of a definite set of biochemical indices, particularly increased respiratory rate and decreased lactic acid content, the latter phenomenon being interpreted to indicate decreased glycolysis. By contrast, during the periods of low sensitivity respiratory rates are lower and lactic acid content higher. It has been claimed that during the development of fish embryos, including those of sevriuga and osetr, several critical periods could be detected and that, accordingly, there occurred periodical, and opposite in sign,

changes in respiratory rates and in lactic acid content under aerobic conditions [17, 20, 21].

No such variations in respiratory activity (sevriuga and osetr eggs), or in lactic acid content under aerobic conditions (sevriuga eggs), could be detected in the present work in the case of developing sturgeon eggs throughout the period of development. Furthermore, in disagreement with the results quoted, in no instance in the present work did the respiratory rate of any batch of eggs fall below the level reached in the preceding period.

It also follows from other data presently at our disposal, that although no sharp changes in the respiratory activity could be observed at the gastrulation stage, this stage in the development of the sturgeon eggs well deserves the status of a special turning point. Thus, at this stage occurred certain shifts in the rates of P^{32} incorporation into some phosphorus-containing fractions and, particularly, there was a marked acceleration of phosphorus renewal in the inorganic and labile phosphorus fractions, a fact apparently reflecting the elevated energetic development of the embryo [1]. This was also borne out by some of our preliminary results, according to which the development of sevriuga eggs proceeded quite normally outwardly in the absence of oxygen up to the late blastula stage, while during gastrulation itself, and the subsequent stages, oxygen was found to be obligatory. In this respect also the present material was found to be similar to the eggs of tailless amphibians whose development up to the gastrulation stage was found to proceed normally in a nitrogen atmosphere in the presence of azide [33], and even in the presence of cyanide [12], while gastrulation itself was completely inhibited under those conditions. DNA synthesis with the concomitant increase of radiophosphorus incorporation into this fraction also begins during gastrulation [1]. Finally, from the morphological point of view, gastrulation is well known as the turning point in the embryonal development of animals.

Thus, in many aspects, gastrulation was found to be a special stage in the development of sturgeon eggs. However, while certain qualitative shifts undoubtedly take place in the further development of these eggs, presently available data are insufficient for the establishment of the existence of other turning points during this process. The changes of other biochemical indices during development failed to show time-coincidence. Thus, sudden changes in the activity of alkaline phosphatase and APP-ase were observed during stages 26-28 while the activity curve of acid phosphatase showed no variation in this period nor was there any change in the progress of the respiratory rate. The reason for these phenomena may rest in the fact that after the gastrulation period it is difficult to encounter in the developing embryo a specific period during which the entire unit becomes immersed in active growth or differentiation. These processes take place in the various organs of the embryo quite asynchronously (particularly toward the end of its development), and the investigation of its metabolism on the whole leads to results which represent a mean sum of the various activities of the individual tissues [34, 35].

SUMMARY

The respiratory activity, intensity of glycolysis and the activity of certain enzymes concerned in phosphate metabolism were investigated in the developing eggs of the sturgeon family (beluga, osetr and sevriuga).

Acid β-glycerophosphatase and adenosinepolyphosphatase (apyrase) showed considerable activity in the non-fertilized mature eggs. At the pH value, at which the acid phosphatase showed maximal activity, there could be demonstrated from the onset of development a maximal activity of an enzyme which split inorganic phosphate from endogenous substrate, predominantly phosphoprotein. This enzymatic activity did not change appreciably throughout the period of development. The maximal activities of the alkaline β-glycerophosphatase and of the enzyme which split endogenous substrates under alkaline conditions were observed at an indentical pH value. At the beginning of the developmental period the activity of the alkaline β-glycerophosphatase was either very small or zero. The activity of this enzyme and particularly that of adenosinepolyphosphatase showed a rapid increase from the stage where active growth of the embryo commenced, accompanied by the formation of specific tissues and organs.

The respiration of the sturgeon eggs was weak in the beginning of the developmental period, but increased throughout the development. The rate of increase, however, was not constant, resulting in the respiration curves assuming a sigmoid shape. It was not possible to demonstrate definite inflexion points on these curves at any specific point of development. The oxygen consumption of sevriuga eggs was found to be, at the beginning of development, 2.5-3.0 μl per 10 eggs per hour; this value reached 12.5-13 μl per 10 eggs per hour at hatching.

Aerobic glycolysis in the developing sturgeon eggs was low throughout the period, nevertheless it constituted an appreciable proportion of the energetic balance of the embryo in the initial stages of development prior to gastrulation.

Considerable analogy between the developing eggs of the sturgeon and the tailless amphibians was observed in the developmental pattern regarding changes in respiration, aerobic glycolysis and the activity of certain enzymes.

Received August 15, 1957

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OXIDATIVE TRANSFORMATIONS OF AMINO ACIDS DURING THE ACTION ON CABBAGE TISSUES OF THE FUNGUS BOTRYTIS CINEREA

B. A. Rubin and T. M. Ivanova

A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR, Moscow

At the present time it is considered that the resistance of plant organisms to infection depends to a considerable degree on the oxidative processes which occur in their tissues [1-4].

In the tissues of resistant forms of plants the oxidative processes and respiration as a whole are activated in response to infection, while in the non-resistant forms these processes either change slightly or there is even a suppression of oxidative processes. It has also been established that the increase in respiration in some forms of resistant plants under the influence of parasites occurs by activation of the enzymes of the so-called residual respiration [5,6]. The presence in plants of flavin enzymes, or, as they are called, enzymes of residual respiration, has been shown in a whole series of studies on different objects [5-10]. As is known, glucose oxidase, aldehyde oxidase, and amino acid oxidases belong to this group. The first of these is not found in plants, while enzymes which oxidize aldehydes (xanthine oxidase) have been found in a number of objects [4, 11, 12]. The data which relate to amino acid oxidases in the literature are very contradictory. A number of authors deny the presence of specific amino acid oxidases in the tissues of higher plants, while other investigators maintain exactly opposite views on this question [4, 7, 13-19].

Starting from our previous findings of the participation of flavin enzymes in protective reactions of plants [4,6], we decided to study which of the components of this group of oxidases here played an active role. In this work we give the results of a study of the change in amino acid oxidase activity in cabbage when the tissues of this plant are acted upon by the fungus Botrytis cinerea.

The work was carried out on two types of white cabbage distinguished by their resistance to Botrytis cinerea. The "Amager" cabbage was strongly resistant to Botrytis, while the "First Number" cabbage belonged to the easily attacked form. Both types of cabbage were grown and kept in the Fungus Selection Station.

In her work, Artsikhovskaia, using the Van Slyke method, did not find free amino acids in cabbage tissues [20]. When we began to study the role of amino acid oxidases on plant resistance we wished first to submit this problem to further study, using the method of paper chromatography.

Among the large number of methods now available for the quantitative determination of amino acids [21] we chose the method of Fischer and Dorfel [22], which we used in a somewhat modified form. For the removal of free amino acids from cabbage tissue we used the method of Dent and co-workers [23].

The chromatographic distribution of amino acids on the paper was determined by the method of Keil' [24]. The results of the determination of free amino acids are given in Table 1.

Both the "Amager" and the "First Number" gave 11 amino acids and the amide, asparagine. Aside from this, the types of cabbage studied differed sharply in the amount of free amino acids contained in their tissues; the resistant type contained five times as many as the non-resistant. The increased intensity of residual respiration noted by Chetverikova [5] in the tissues of "Amager" cabbage probably explains the higher content of amino acids.

TABLE 1

Content of Free Amino Acids in the Tissues of Different Types of Cabbage

In mg per 100 g fresh weight of tissues

Amino acids	Type of cabbage		Amino acids	Type of cabbage	
	"Amager"	"First Number"		"Amager"	"First Number"
Aspartic acid	51.9	7.6	Valine	30.0	16.3
Glutamic acid	57.8	12.7	Leucine	11.6	trace
Glycine	47.8	7.2	Histidine	15.5	15.5
Alanine	102.3	11.8	Arginine	16.2	
Serine	87.7	14.2	Proline	30.0	trace
Tyrosine	18.0	trace	Asparagine	42.9	14.9

It is interesting that in the experiments of Mukhin [25] on the red cabbage "Stone Head" it was more resistant than the white cabbage "Moscow Glory." According to the data of Tsonev [26], who determined some of the amino acids in these cabbages by a chemical method, the red cabbage contained two to three times more free amino acids (histidine, arginine, tyrosine, methionine) than the white cabbage.

TABLE 2

Amino Acid Content in the Tissues of Healthy and Infected "Amager" and "First Number" Cabbages

In mg per 100 g fresh weight of tissues

Amino acid	Healthy tissue (control)	Infected tissue, distance from point of infection in mm		
		0–10	10–20	30–40
Amager				
Aspartic acid	51.9	53.0	43.7	45.8
Glutamic acid	57.8	44.1	50.6	52.0
Serine	87.7	58.3	72.1	77.2
Glycine	47.8	36.2	44.7	57.0
Alanine	100.1	63.5	72.1	77.2
Total content of studied amino acids	345.3	255.1	280.2	330.7
Loss of amino acids in 5 days	—	90.2	65.1	15.3
First Number		0–5	5–10	10–15
Aspartic acid	7.6	7.2	6.6	6.7
Glutamic acid	12.7	7.9	7.9	6.2
Serine	14.2	6.3	7.6	6.7
Glycine	7.2	5.8	5.8	6.4
Alanine	11.9	9.5	6.6	6.6
Total content of studied amino acids	53.6	36.7	33.5	33 6
Loss of amino acids in 4 days	—	16.9	20.1	20.0

To explain the role of oxidative transformations of the amino acids in protective reactions of cabbage we studied the effect on amino acid oxidase activity of infection by Botrytis.

Infection of the tissues was produced by the method which we described earlier [4,20]. The activity of amino acid oxidase was determined by the change in content of the amino acids of healthy and infected tissues. The determination was carried out at different distances from the point where the infection was introduced.

The results from one of the experiments are given in Table 2.

The data of Table 2 show that there is a very decided change in amino acid content connected with oxidation in the tissues which are close to the point of infection (0-10 mm) and this reaction occurs most strongly in the "Amager" cabbage, which is resistant to infection. In the tissues of this variety near the place of infection 90 mg of amino acids per 100 g of tissue are used up in 5 days, while in the "First Number" only 20 mg are destroyed. Hence, the activity of the amino acid oxidase in the infected "Amager" tissues is four to five times higher than in the "First Number".

It is interesting to compare the change in total amino acids determined in different layers of cabbage tissue with the change in intensity of residual respiration in the same layers, as found by Rubin and Chetverikova [27] in analogous experiments (Fig. 1).

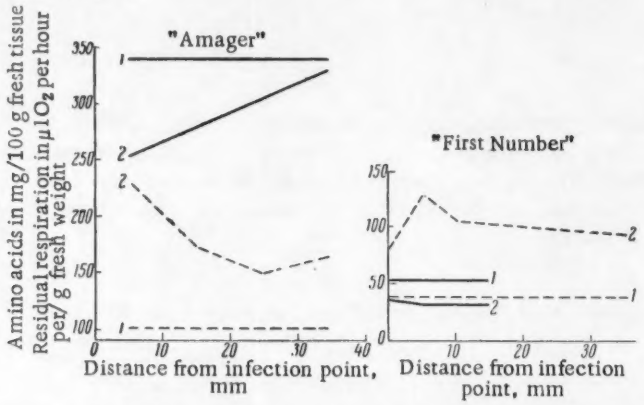
Amino acids in mg/100 g fresh tissue
Residual respiration in μl O_2 per hour per g fresh weight
"Amager"
"First Number"
Distance from infection point, mm
Distance from infection point, mm

Fig. 1. Change in intensity of residual respiration and amino acid content in cabbage leaves infected with Botrytis cinerea
——— Amino acid content, — — — Residual respiration;
1) control; 2) experimental.

Figure 1 shows that when the cabbages are infected with Botrytis the residual respiration in both types is activated and the maximum such respiration is found not in the living tissues which touch the tissues killed by the toxin, but at some distance. According to our data, it is in just this section of the tissues that maximum amino acid oxidation occurs. As the distance from the point of infection increases, the residual respiration falls, and there is a corresponding decrease in consumption of amino acids. This relation is found especially clear in the "Amager" cabbage, whose tissues, which have a more intense residual respiration, respond to an external parasite by greater activation of this respiration.

The opposite character of the change in intensity of residual respiration and the amino acid content allows us to assume that under the influence of Botrytis the so-called residual respiration in cabbages occurs through participation of amino acid oxidases. This is also indicated by the fact that in the "Amager" tissues close to the point of infection, ammonia is found in amounts which average 270 μl per g of fresh tissue. Ammonia was determined by the microdiffusion method [28]. In the "First Number" cabbage such an effect was not found, which fully agrees with the results which show that Botrytis infection in "First Number" tissues causes only slight oxidation of amino acids (Table 2).

The formation of ammonia at the boundaries of the infected tissue in the resistant "Amager" variety is one of the links in the chain of protective reactions of the cabbage against Botrytis. It is very probable that the observation first made by Artsikhovskaia [20] of the formation in the resistant type of cabbage of a layer of necrotized cells is due to the accumulation of ammonia. The latter, as is known, is a strong poison for the cells of higher and lower plants. The enrichment of the cells of the host plant by ammonia serves as a chemical barrier which hinders further spread of the infection.

These results indicate that the Botrytis-resistant "Amager" type of cabbage not only has a high amino acid oxidase activity, but can also increase this activity greatly in response to infection. The non-resistant type,

"First Number," has a low amino acid oxidase activity and does not increase it on infection.

It is known that when amino acids are deaminated with amino acid oxidase, hydrogen peroxide is formed and can be used in the plant tissues to oxidize other substances with the aid of the enzyme peroxidase. Chetverikova showed [4] that the value of the residual respiration in cabbages changed in parallel with the change in peroxidase activity. According to our experiments (Table 3) the peroxidase activity* increases sharply under the influence of Botrytis infection only in the "Amager," in tissues which lie close to the point of infection. In the "First Number" cabbage peroxidase activity in infected tissues is somewhat lower than in the controls.

TABLE 3

Effect of Infection on Activity of Peroxidase of Cabbages in Relative Units

Type of cabbage	Healthy tissue (control)	Infected tissue	
		Distance from point of infection in mm	
		0 – 10	10 – 20
"Amager"	18.55	32.45	21.64
"First Number"	9.73	6.83	5.90

Parallel measurements of peroxidase activity (Table 3) and amino acid oxidase in "Amager" (Table 2) at the borders of the infection show a functional relation between these enzymes.

It is important to explain the initiation of amino acid oxidase activation at the border of the infected tissue in the resistant type of cabbage. One of such initiators may be the enzyme of the fungus Botrytis cinerea.

Table 4 shows that the mycelia of this fungus actually contain active amino acid oxidase.

TABLE 4

Oxidation of Amino Acids by Mycelia of Botrytis cinerea

In μl per 1 g tissue in 3 hours

Amino acid	Control	Experiment
Glutamic acid	162	189
Aspartic acid	162	176
Glycine	162	200
Serine	162	188

Further study of the problem of participation of fungus amino acid oxidase in the oxidation of cabbage amino acids was carried out by infiltration of Botrytis cinerea toxin into the cabbage tissues and by observing the effect of the toxin on the amino acid content. According to the data of Rubin and Chetverikova [27] cells injured by the toxin of Botrytis cinerea contain no oxidative enzymes. The determination of amino acid content was carried out 1, 3, 4 and 5 days after infiltration of the toxin.

The results shown in Fig. 2 show that infiltration of the toxin causes the strongest effect on the tissues of the resistant type "Amager". As in the infection by fungus cultures, infiltration of the toxin sharply increases the oxidation of amino acids. During the first day under the influence of toxin infiltration, serine is most actively oxidized; then its oxidation decreases and on the third day becomes slight. The maximum oxidation of alanine occurs on the third day. Oxidation of aspartic and glutamic acids during the first three days remains about constant.

It is interesting that on the fifth day the amino acid content of the samples which were infiltrated by toxin rose considerably. The origin of this increase may be the splitting of the cabbage proteins by the toxin.

Figure 2 also shows that in the "Amager" cabbage the change in aspartic acid content differs greatly from the change of the other amino acids. In the first four days of the experiment the serine, alanine, and glutamic acid content fell in the controls and in the experimental samples, while the aspartic acid content during this time, on the contrary, increased. It is very likely that both these effects are interrelated. We now know that oxaloacetic and α-ketoglutaric acids take part in the Krebs cycle and occupy a central position in

*Peroxidase activity was determined by the colorimetric method of Boiarkin [29].

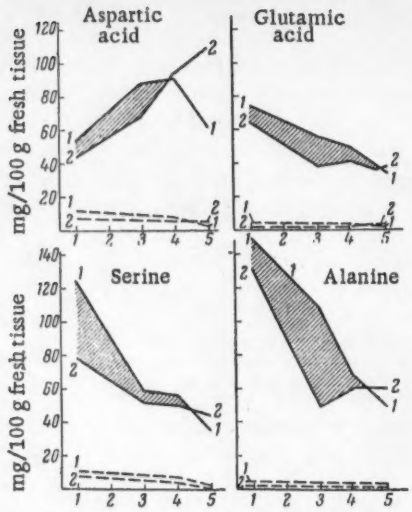

Fig. 2. Effect of botrytis cinerea toxin on the amino acid concentration in cabbages: —— "Amager," – – – "First Number." 1) Control; 2) experimental. Shaded portion – Amino acids used up.

the nitrogen metabolism of plants [31]. It is also known that aspartic acid is easily formed from other amino acids. For example, recently Sivaramakrishnan and Sarma [32], using labeled atoms, showed that aspartic acid is formed from glutamic acid in pea sprouts. Also, according to our data, cabbage tissues contain asparagine (Table 1), from which aspartic acid can be formed.

Thus, the data show that infiltration of Botrytis toxin causes changes in cabbage tissues analogous to those which occur under the influence of the fungus itself. Hence the findings in infected cabbages of activation of amino acid oxidase cannot be ascribed to the enzymes of Botrytis but relate to the reaction of the tissues of the host plant to deprivation of enzyme activity by the Botrytis toxin.

It remains to explain our findings of a difference in ability of the cabbage tissues to oxidize the individual amino acids. Experiments showed that tissues of the resistant type "Amager" are able to oxidize actively most of the amino acids of our study (we used 21 amino acids) with the exception of tyrosine, serine, and histidine, which hinder the absorption of oxygen. The dicarboxylic acids, and also glycine, proline, and threonine are expecially well oxidized.

Analogous data were obtained by Boswell [13] for turnip sections. In distinction to the "Amager" cabbage tissues, the tissues of the non-resistant "First Number" oxidized valine, threonine, and isoleucine only slightly; the other amino acids caused varying degrees of inhibition of respiration in the cabbage tissues.

TABLE 5

Effect of pH on Amino Acid Oxidation by Sections of "Amager" Cabbage

In μl O_2 per 1 g tissue after 3 hours

Amino Acid	pH 5.3			pH 6.2			pH 7.1		
	Control	Experimental	Difference	Control	Experimental	Difference	Control	Experimental	Difference
Glutamic acid	56	86	30	95	99	4	267	259	– 8
Aspartic acid	56	85	29	95	113	18	267	316	49
Serine	41	26	– 15	95	94	– 1	267	296	29
Tyrosine	41	32	– 10	95	39	– 56	267	258	– 9

The amides of dicarboxylic acids are not oxidized at all by the cabbage sections. According to the data of Kretovich and Drozdova [16] asparagine and glutamine are also not oxidized by suspensions of rye sprouts. It is interesting that serine, which in the previous experiments was well oxidized by cabbage tissues (Fig. 2) in these experiments hindered respiration of these tissues. This fact may be connected with the pH change produced in cabbage tissues under the influence of infection (Table 5).

Table 5 shows that different amino acids are oxidized differently by cabbage sections depending on the pH of the medium. Glutamic acid is well oxidized only in an acid medium; in a neutral medium this amino acid hinders somewhat the respiration of the cabbage section. Aspartic acid is a good substrate for cabbage amino acid oxidase; it increases the absorption of oxygen at all the pH values which we studied. Tyrosine, on

the contrary, hinders respiration of cabbage tissue at all pH values studied by us. Serine is oxidized by cabbage sections only in neutral medium; in acid medium (pH 5.3) it suppresses oxygen absorption by cabbage tissue.

Thus, oxidation by different amino acids depends on the pH of the medium.

The material obtained in our work confirms the results of our previous investigation according to which a system of flavin enzymes, peroxidases, should play an important part in the immunity reactions of cabbage toward Botrytis.

SUMMARY

Tissues of varieties of cabbage, resistant or non-resistant to the fungus Botrytis cinerea, are very different in free amino acid content: the first has five to six times more than the second.

Under the influence of infection the enzymatic oxidation of amino acids with formation of corresponding amounts of ammonia is greatly increased in the resistant type. Parallel with the activation of amino acid oxidase in the resistant variety of cabbage there is activation of the associated enzyme, peroxidase.

The combination of these factors allows us to assume that amino acid oxidase shares in the protective reactions of cabbage tissues against Botrytis cinerea.

The authors express their thanks to M. A. Davydova for participating in the experimental part of the work.

Received September 19, 1957

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THE SUBMOLECULAR STRUCTURE OF GLYCININ AND THE CONDITIONS FOR ITS REVERSIBLE ASSOCIATION

V. L. Kretovich, T. I. Smirnova and S. Ia. Frenkel'

A. N. Bakh Institute of Biochemistry (Moscow) and Institute of High-Molecular Compounds (Leningrad), Academy of Sciences, USSR

In a previous communication [1] based on the study of ultracentrifuge "fractions" of soy globulin obtained by precipitation with various concentrations of ammonium sulfate, we expressed the idea that glycinin, like edestin and some other plant globulins, could show a variable molecular weight depending on the conditions of its solution.

Such behavior means that the kinetic units ("macromolecules") of glycinin consist of identical submolecules which are capable of reversible association; the degree of this association should be determined by the nature of the surrounding medium and the steric ("packing") factors [2].

The probable existence of an equilibrium between the kinetic units with different degrees of association is indicated by the following facts, which were given in the previous work [1,3].

1. Neither we nor Naismith [4] ever succeded in obtaining a "stable" fraction by fractionation.

2. Depending on the conditions of solution, additional components were found in greater or less amount in the "fractions," and solvents could always be chosen in which the "fractions" were identical with the starting protein.

3. In the previous experiments, which were carried out without sufficiently careful control of the effects of concentration (the experiments were performed at protein concentrations of 1%), we found three fundamental components which we named conditionally on the basis of sedimentation coefficients S_8 , $S_{10.7}$, and S_{14}- components. The calculation of the molecular weight of these components showed that they existed in simple ratios among themselves (1 : 2 : 3). When the composition of the solvent was changed, these components could vanish or again reappear, and the loss of one component was always equal to the increase of another component, etc.

In this study we decided to investigate these effects in detail within wide pH limits and various ionic strengths, and with consideration of possible effects of concentration. The fundamental purpose of the study was an investigation of the submolecular structure of glycinin and an explanation of the conditions for its reversible association.

EXPERIMENTAL

Obtaining the Protein

Powdered soy cotyledons, defatted with ether, were extracted with 10% NaCl overnight at 4°. The resulting solution was centrifuged and filtered through gauze, then saturated with ammonium sulfate. After the solution was centrifuged, the protein which was precipitated by ammonium sulfate was dialyzed against distilled water until reaction for chloride and sulfate ions disappeared. The globulin which formed during dialysis was separated from albumin by centrifuging and repeated washing with twice-distilled water. The resulting protein

was dried by a lyophilic method (initial protein), and a second portion of protein was fractionated with ammonium sulfate. All these operations were carried out in the cold [1].

The experiments with the ultracentrifuge were carried out under the same conditions as in the previous experiments. The values for the sedimentation coefficients, however, were more exact, since for each series of experiments we determined the viscosity and density of the corresponding solvents, and all the sedimentation coefficients were run at 20° and in pure water, for $S_{20,w}$ which was based on these measurements (and not on tables). In the calculations the correction for partial specific volume V was 0.745 (at 20°), which is characteristic for most plant globulins. All the experiments were run at 50,000 rpm.

RESULTS

The results of all the experiments with different procedures are grouped into six tables for satisfactory analysis. Therefore, separate experiments may appear several times, in different tables, but as will be evident

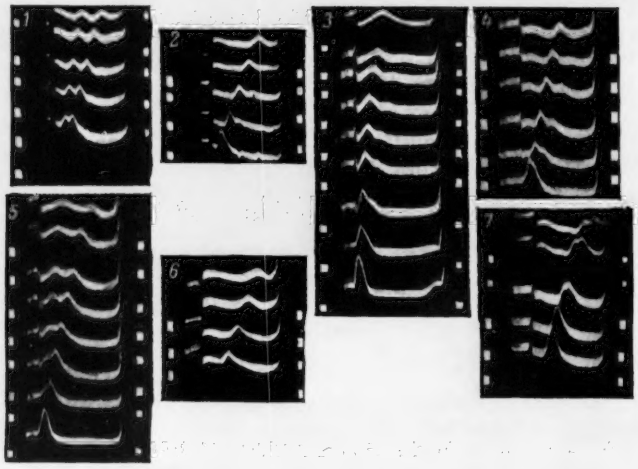

Fig. 1. Sedimentation diagram for soy glycinin. 1) In 10% NaCl (first picture 26 minutes after beginning sedimentation); 2) in borate buffer, pH 9.0; 3) in KCl + HCl buffer, pH 3.0 (seventh print, after 70 min, eighth, 90 min, ninth, 120 min from beginning of sedimentation); 4) in phosphate-citrate buffer, pH 3.4; 5) in phosphate-citrate buffer, pH 3.0; 6) in KCl + HCl buffer, pH 3.0 with later dialysis against phosphate buffer, pH 9.0; 7) in phosphate buffer, pH 9.0 + cysteine (1.0%). The order of the prints on the sedimentation diagrams is always from below upwards; the prints were made each 10 min except as shown. The protein solution was 1%.

below, analysis of the results on the basis of one combined table would be difficult. All the tables are constructed in the same way and additional explanations are not needed. The sedimentation diagrams of all the experiments are given in Figs. 1 and 2. The sedimentation coefficients $S_{20,w}$ are expressed in Svedberg units.

1. Demonstration of the Identity of the "Fraction" and the Starting Protein

In Table 1 we give the results of five pairs of experiments with the starting globulin and fractions 65 — 70, which were obtained at 65 — 70% of saturation. The conditions of solution in each pair were completely identical except in pair V, where the concentrations differed.

We see that with other conditions equal, in the first four pairs there is not only agreement in the amount of components, including even traces of the "extra-heavy" S_{20}-component, but also, within the limits of experimental scattering, of the sedimentation coefficients of these components. The two components of pair III are

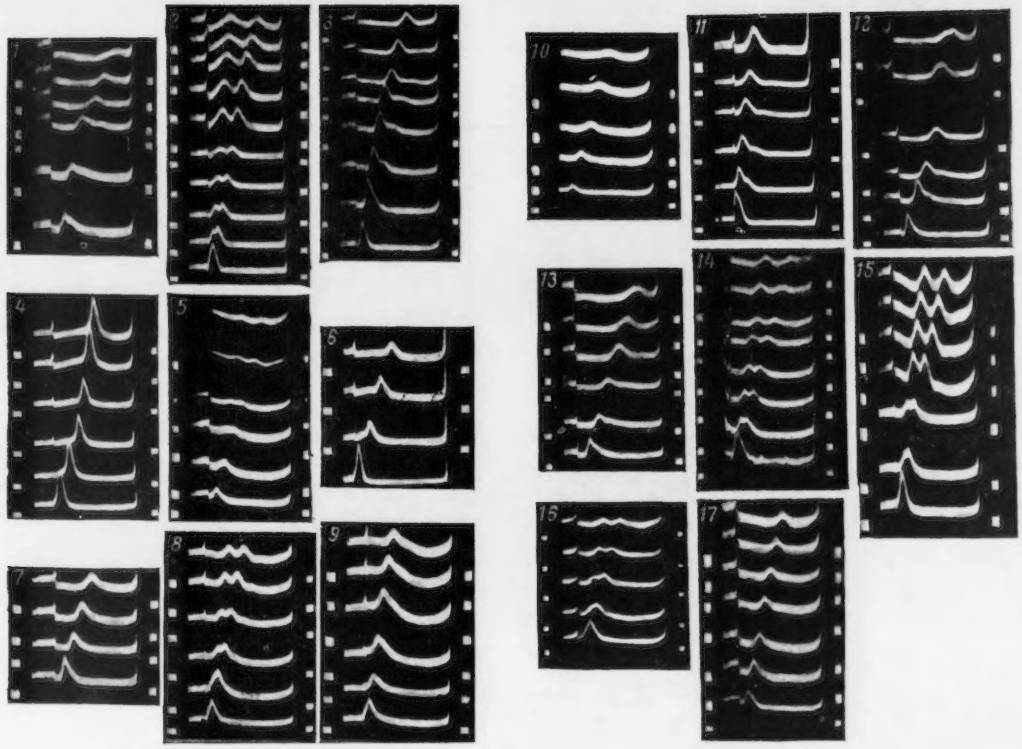

Fig. 2. Sedimentation diagram for fractions 65 – 70. 1) In borate buffer, pH 9.0 (first print after 12 min, second, 20 min, 50 sec, third, 30 min, 55 sec, fourth, 43 min, fifth, 51 min, 45 sec, sixth, 61 min from start of sedimentation; 2) in KCl + HCl buffer, pH 3.0; 3) in phosphate buffer, pH 3.4; 4) 2% solution in phosphate-citrate buffer, pH 3.0; 5) 0.3% solution in 10% NaCl; 6) in phosphate-citrate buffer, pH 3.0; + cysteine (0.3%) (first print 8 min 35 sec, second, 30 min, third 50 min, fourth 70 min after beginning of sedimentation; 7) in phosphate-citrate buffer, pH 3.0, with later dialysis against a phosphate buffer, pH 9.0; 8) in phosphate buffer, pH 4.5 + 10% NaCl; 9) in phosphate-citrate buffer, pH 2.2 + 10% NaCl; 10) in phosphate-citrate buffer, pH 2.2, with later dialysis against a phosphate buffer, pH 9.0; 11) in phosphate-citrate buffer pH 2.2 (first print 10 min, second 30 min, third 50 min, fourth 70 min, fifth, 93 min, sixth, 120 min from start of sedimentation; 12) in phosphate-citrate buffer, pH 7.1; 13) in phosphate buffer, pH 7.7; 14) in a KCl + HCl buffer, pH 3.0 + 10% NaCl; 15) in borate buffer, pH 9 + 10% NaCl; 16) in 10% NaCl; 17) 0.3% solution in phosphate-citrate buffer, pH 3.4.

an exception ($S_{20,w}$ = 8.53 and 6.66), but we have reason to believe that the $S_{6.7}$- component is a denatured form of the "ordinary" S_8-component (for which $S_{20,w}$ itself is close to 9). We return to this question below. As to pair V, the different numbers of the components can be fully explained by the different concentrations (see Table 2), and the lowered value for $S_{20,w}$ (6.66) is due to denaturation or to ordinary concentration relations of S.

Comparison of the sedimentation diagrams corresponding to the separate experiments of Table 1 shows that usually in any pair there is agreement not only in quantity but also in relative content of the components (the area of the corresponding peaks is about the same).

2. Effects of Concentration

In Table 2 we give three groups of experiments, from which we can draw the following conclusions:

a) With other conditions equal, the $S_{20,w}$ S_{14}-components depend greatly on concentration (group I).

b) For the S_8-component this relation at pH > 3 is almost absent (comparing group III with pair V of Table 1, the reservation made above must be considered).

TABLE 1

Identity of "Fractions" and Starting Protein

Protein concentration 1%

Number in corresponding figure	Experiment No.	Protein	Solvent	Number of components	S constants				
					1	2	3	4	5
1,1	I	Starting globulin	10% NaCl	2	—	8.70	—	14.19	—
2,1		Fraction 65-70	The same	2	—	9.09	—	13.82	—
1,2	II	Starting globulin	Borate buffer, pH 9.0	1	—	—	—	13.86	trace 22
2,1		Fraction 65-70	The same	1	—	—	—	13.42	trace 22
1,3	III	Starting globulin	KCl + HCl, pH 3.0	2	3.08	8.53	—	—	—
2,2		Fraction 65-70	The same	2	2.87	6.66	—	—	—
1,4	IV	Starting globulin	Phosphate-citrate buffer, pH 3.4	1	—	9.19	—	trace 15.03	—
2,3		Fraction 65-70	The same	1	—	8.74	—	trace 15.47	—
1,5	V	Starting globulin	Phosphate-citrate buffer, pH 3.0	2	3.14	8.22	—	—	—
2,4		Fraction 65-70*	The same	1	—	6.96	—	—	—

*Protein concentration 2%.

TABLE 2

Different Protein Concentrations

No. of corresponding figure	Expt. No.	Protein	Protein conc. %	Solvent	No. of components	S constants				
						1	2	3	4	5
2,1	I	Fraction 65-70	1	10% NaCl	2	—	9.09	—	13.82	—
2,5		The same	0.3	The same	2	—	9.28	—	15.28	—
2,3	II	"	2	Phosphate-citrate buffer, pH 3.4	2	—	8.87	—	trace 14.17	—
			1	The same	2	—	8.74	—	trace 15,47	—
			0.3		1	—	8.69	—	—	—
	III	Starting globulin	2	Phosphate-citrate buffer, pH 3.0	1	—	6.96	—	—	—
		The same	1	The same	2	3.14	8.22	—	—	—

c) In solutions with small ionic strength, decrease in concentration leads to dissociation of the heavy components (gradual disappearance of the S_{14}-component in group II) and the appearance of light components ($S_{20,w} = 3.14$ in group III).

Thus the concentration usually affects the equilibrium in the reversibly associated system of soy globulin. However, as will be seen below, this effect is not definite, and the limiting number of components depends greatly on electrostatic factors.

3. Role of pH

In Table 3 we give results of experiments carried out in different buffers with about the same ionic strength ($\mu \approx 0.1 - 0.2$), over the pH range 9.0 – 2.2. In the range pH 4.0 – 7.0 experiments were impossible since near the isoelectric point (pH ≈ 5.0) the proteins were insoluble.

TABLE 3

Relation to pH

Protein concentration 1%

No. of corresponding figure	Expt. No.	Protein	Solvent	No. of components	S constants				
					1	*2*	*3*	*4*	*5*
2,*1*	1	Fraction 65-70	Borate buffer, pH 9.0	1	—	—	—	13.42	trace
2,*13*	2	The same	Phosphate buffer, pH 7.7	1	—	—	—	14.38	"
2,*12*	3	"	Phosphate buffer, pH 7.1	1	—	trace	—	14.17	—
2,*3*	4	"	Phosphate-citrate buffer, pH 3.4	2	—	8.74	—	trace 15.47	—
2,*2*	5	"	KCl + HCl buffer, pH 3.0	2	2.87	6.66	—	—	—
2,*11*	6	"	Phosphate-citrate buffer, pH 2.2	1	2.36	trace 5.47	—	—	—

The table shows that increased pH permits association. In fact, at pH 9.0, besides the basic S_{14}-component there are traces of an extra-heavy component; at pH 7.7 and 7.1 there is almost pure S_{14}-component, and in the narrow pH range 3.5 – 2.2 the S_{14}-component vanishes and the S_8-component appears and then undergoes either denaturation, which leads to a lowered $S_{20,w}$, or further splitting which finally leads to almost pure light component, with $S_{20,w} = 2.36$.

Probably $S_{20,w} = 2.87$ at pH 3.0 corresponds to the same component; the lowering of the sedimentation coefficient at pH 2.2 can be ascribed to a reversible acid denaturation.

TABLE 4

Reversible Action of pH

Protein concentration 1%

No. of corresponding figure	Expt. No.	Protein	Solvent	No. of components	S constants				
					1	*2*	*3*	*4*	*5*
1,*3*	*I*	*1* Starting globulin	KCl + HCl buffer pH 3.0	2	3.08	8.53	—	—	—
		2 The same	Phosphate-citrate buffer, pH 3.0 with later dialysis at pH 9.0 in phosphate buffer						
1,*6*				1	—	—	—	13.44	—
1,*2*		*3* "	Borate buffer, pH 9.0	1	—	—	—	13.86	traces
2,*11*	*II*	*4* Fraction 65-70	Phosphate-citrate buffer, pH 2.2	1	2.36	traces 5.47	—	—	22.00 —
2,*10*		*5* The same	Phosphate-citrate buffer, pH 2.2, with layer dialysis at pH 9.0 in phosphate buffer	1	—	—	—	11.92	—
2,*1*		*6* "	Borate buffer, pH 9.0	1	—	—	—	13.42	traces

To test our concept of the existence of an equilibrium which depends on pH we must determine the reversibility of pH action. For this purpose we dialyzed solutions which contained only the light component at pH 2.2 and 3.0 in different buffers overnight in the cold against borate and phosphate buffers, pH 9.

Table 4 gives the results of two series of such experiments from which it can be seen that after dialysis the light component regularly disappears and there is reappearance of the S_{14}, and in two cases, the S_{22} component. However, the value of $S_{20,w}$ for the S_{14} component in experiment II is somewhat low.

It would also be interesting to study the reversible association in repeated cycles of pH 2.2 — 9 — 2.2.

The direction of dissociation in relation to pH permits us to draw the following conclusions. Evidently reversible dissociation in this system depends on the antagonism of van der Waals and electrostatic forces, on which the corresponding attraction or repulsion of the submolecules depends. Below the isoelectric point ionization of the basic group occurs, leading to accumulation on the surface of the submolecule of free positive charges and the occurrence of coulombic forces of repulsion; there is also indirect evidence of configurational changes in the submolecule which can lead to alterations in steric conditions which permit association. On the whole, the van der Waals forces are suppressive and produce dissociation to submolecules. We measured the coefficient of diffusion of fraction 65 — 70 under conditions of experiment 4 (Table 4) where we could neglect the traces of the component with S = 5.47. It showed $D_{20,w} = (11.37 \pm 0.50) \cdot 10^{-7}$ cm^2/sec. In combination with $S_{20,w} = 2.36 \cdot 10^{-13}$ cgs and $V_{20,w} = 0.745$ (as for most plant globulins), this leads to a molecular weight M = 19,800. An analogous value was obtained by Johnson and Naismith [5] for the submolecule of arachin, although in this case complete dissociation was obtained by the action of urea or guanidine. Dissociation also proceeded through formation of intermediate components with $S_{20,w} \approx 8$ (with a starting 1% arachin solution $S_{20,w} = 14$).

Evidently, at an alkaline pH configurational changes in the submolecule are eliminated and the NH_3^+ ion disappears from their surface. Ionization of the COOH group is ineffective, perhaps because of its reaction with the surrounding Na^+ cations or with basic groups on the surface of the macromolecule.

In this connection, it is characteristic that moderate ionization of the carboxyl at pH 9 is not especially reflected in the hydrodynamic properties of serum albumin (with isoelectric point near pH 5), while on the acid side, even much closer to the isoelectric point (at pH 3) there are already strong configurational changes in globulin. We could give other examples of such apparent "inequalities" in NH_3^+ and COO^- ions on the surface of protein molecules.

4. Effect of Solvent Composition and Ionic Strength

It is impossible to conclude from the previous data that the composition of the buffer, with other conditions equal, has any effect on the reversible association of glycinin. In other words, if specific binding of anions on the surface of the macromolecule occurs, it does not affect the equilibrium.

On the other hand, increasing the ionic strength by adding NaCl (to 10%) sharply changes the equilibrium, as Table 5 shows. Two conclusions follow from Table 5:

1. Addition of NaCl increases the stability of the S_8-component, preventing its dissociation in acid pH and partly preventing its association in alkaline pH.

2. NaCl permits increased solubility of the protein as a whole, since with it we can obtain solution closer to the isoelectric point (experiment 7, Table 5). It is true that such solutions contain an anomalous S_{11}-component.

The fact that NaCl prevents dissociation in acid pH is not surprising. Around the positive NH_3^+ ions responsible for this dissociation there is a thick cloud of negative ions which screen the positive charges and thus shift the equilibrium between coulombic and van der Waals forces toward the side of the latter.

As to the partial prevention of association at pH 9 by the same NaCl, we should consider that the already mentioned "ineffectiveness" of the ionization of the COOH group is chiefly due to the electrostatic reaction (attraction) with the basic groups on the surface of the submolecule. The same screening effect of the opposite ions is responsible for part of the dissociation.

TABLE 5

Effect of Solvent Composition and Ionic Strength

Protein concentration 1%

No. of corresponding figure	Expt. No.	Protein	Solvent	No. of components	S constants				
					1	2	3	4	5
2,1	I	1 Fraction 65-70	Borate buffer, pH 9	1	—	—	—	13.42	trace
2,15		2 The same	Borate buffer, pH 9 + 10% NaCl	2	—	7.64	—	13.17	—
2,2	II	3 "	KCl + HCl buffer, pH 3	2	2.87	6.66	—	—	—
2,14		4 "	KCl + HCl buffer, pH 3 + 10% NaCl	2	—	7.92	—	13.71	—
2,11	III	5 "	Phosphate-citrate buffer, pH 2.2	1	2.36	trace	—	—	—
2,9		6 "	Phosphate-citrate buffer, pH 2.2 + + 10% NaCl	1	—	5.47 8.35	—	—	—
2,8		7 "	Phosphate buffer, pH 4.5 + 10% NaCl	2	—	7.26	11.28	—	—

This result is open to other interpretation: at pH 9 specific adsorption of the Na^+ ions may occur, and the forces of repulsion which arise between them may lead to partial decomposition of the S_{14}-component.

There is an analogous interpretation for the low solubility of the protein at the isoelectric point which is overcome at increased ionic strength. If this low solubility is due to the reaction of the COOH and NH_2 groups, then screening these by opposite ions or binding the cations should lower the effectiveness of this reaction and raise the solubility.

We can now define more accurately our conclusions as to the reason for the reversible association. It is due to the antagonism of the coulombic and van der Waals forces below the isoelectric point and their concurrence above the isoelectric point. Therefore, increased ionic strength below and above the isoelectric point leads to opposite results.

The more probable of the two possible mechanisms for the action of NaCl on the equilibrium is the simple screening of charges by opposite ions, since for specific binding of small ions a great concentration is not essential [6].

5. Complete Number of Components. Effect of Cysteine. Possible Difference of the "Fraction" from the Starting Protein

On the basis of the above results we can affirm that in the pH interval 2.2 — 9 there are four stable modifications of glycinin which occur in equilibrium with each other. These are the submolecule with molecular weight ~ 20,000 and $S_{20,w}$ = 2.4 — 3.1 (depending on pH), the S_8-component (M = 126,000), the S_{14}-component (M = 380,000), and the "extra heavy" S_{22}-component.

The existence of a limited number of stable associated components unavoidably indicates the existence of intermediate forms. We can say that such forms are absent between the S_{14}- and the S_{22}-components. On the other hand, transformation from the S_8- to the S_{14}-component is possible through the intermediate component with $S_{20,w} \approx 11$. The presence of a range of sedimentation coefficients from 5.5 to 8 below the isoelectric point also raises the question of the existence of intermediate components between the submolecule and the S_8-component.

If we admit that such intermediate components exist, we should consider them very unstable since in comparison to the four basic components, they appear unsystematically in the sedimentation system, and only under special conditions.

Thus, for example, the S_{11}-component, which appears as a double of the S_8-component and is considerably more asymmetric than the S_8- or S_{14}-components [3], appears only in the presence of cysteine (experiment 3, Table 6 and [4]), or when the protein dissolves close to the isoelectric point at high ionic strength (experiment 7, Table 5).

TABLE 6

Action of Cysteine

Protein concentration 1%

No. of corresponding figure	Expt. No.	Protein	Solvent	No. of components	S constants 1	2	3	4	5
1,7	1	1 Starting protein	Phosphate buffer, pH 9.0 + 1.0% cysteine	1	—	—	10.53	—	—
1,2		2 The same	Borate buffer, pH 9.0	1	—	—	—	13.86	trace 22.0

The action of cysteine is reminiscent of that of NaCl in three respects: first, even small addition raises the solubility of the protein as a whole; second, as is clear from Table 6 and [1] it permits dissociation of the S_{14} - component to the S_8 - component or prevents full association of the S_8 - to the S_{14} - component; third, this action is lost after dialysis. The latter fact also shows that compounds of cysteine with the protein are very labile.

Probably the direct action of cysteine on the protein also causes modification of the electrostatic forces [1] as a result of which there is a change in the balance between them and the van der Waals forces. In distinction to NaCl, however, here we can also assume specific binding of cysteine by the protein. If this union has an ionic character and is due to the – SH group (which is very likely, since the other amino acids have a much weaker peptizing action on proteins) then the ionization of the other carboxyls may cause coulombic repulsion which prevents combination with the double of a third molecule of the S_8 - component needed to form the S_{14} - component.

The existence of intermediate components between the S_8 - component and the submolecule is unlikely, both by analogy with arachin and on the basis of the hypothetical mechanism of association given below.

In our view, it is characteristic that the molecular weight of components heavier than the S_8 - component are multiples of M for the S_8 - component which indicates the basic stability of this modification of glycinin. The formation of S_8 - from submolecules can occur as follows.

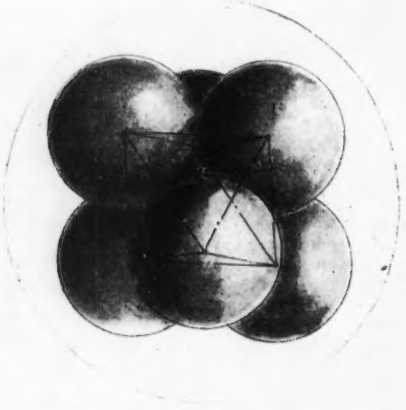

Fig. 3. Schematic representation of the glycinin macromolecule.

The submolecules are so grouped (Fig. 3) that they are isolated from the aqueous medium by hydrophobic particles. It is natural that full isolation requires some minimum of submolecules, in this case, from an estimate of the molecular weight, six. Since any other combination with $n < 6$ is unsaturated, it is unstable and tends to saturation by changing to the S_8 - component.

By virtue of this specificity and by analogy with the ordinary globular proteins composed of polypeptide chain or chains joined through – S – S – bridges, the S_8 - component is capable of a deep change in configuration, depending on pH, which precedes the split into submolecules.

In the association of the S_8 - component the van der Waals forces also play a smaller part. It is even the case that the heavier components do not undergo much configurational change and when conditions are altered, merely dissociated into the S_8 - component.

This could be shown directly if the protein would dissolve in a mixture of dioxane and water. By analogy with insulin [7], we would then expect full splitting of the protein into submolecules.

Thus, we are inclined to ascribe the peak in the sedimentation diagram at $S_{20,w} = 5 - 7$ to a reversible acid denaturation of the S_8-component.

We may add the following to this. The split of the S_8-component when the pH is lowered is made easier by the configurational change which we mentioned. At the same time as the accumulation of positive charges, which "break up" the globulin, there occurs by the same action a deformation (swelling) of the submolecule which finally leads to a steric hindrance to its combination in the globulin. Therefore, we can expect that simple denaturation of the S_8-component by urea or guanidine can give the submolecule even at pH values at which the S_8-component is stable. Probably this is just what happens in the case of arachin [5].

Finally, we turn to the question of the systematic decrease of the $S_{20,w}$ S_8-component of fraction 65 – 70 compared to the starting globulin. It is quite possible that when it is precipitated by $(NH_4)_2SO_4$ the protein loses a small amount of some admixed substance (which could even be the ions of a divalent metal) which had protected the configuration of the submolecule by forming an additional "supporting" bond [2]. Therefore the S_8-component of the "fraction" would differ from the S_8-component of the starting globulin only in its more labile configuration.

DISCUSSION

The name given by Osborne and Campbell to the fraction of protein which is precipitated on dialysis of a 10% NaCl extract of defatted soy meal, glycinin [10], should be placed in quotation marks on the basis of the most recent studies of the method of electrophoresis and the ultracentrifuge. Thus, Briggs and Mann [8] in an electrophoretic study in a phosphate buffer, pH 7.6, I = 0.1, with glycinin obtained according to Osborne and Campbell, and also by the use of several other methods, showed that different preparations of "glycinin" were not electrophoretically homogeneous. They obtained an electrophoretically homogeneous globulin by cooling a concentrated aqueous extract of soy meal. They showed that this protein is composed of part of the "glycinin." However, experiments on solubility showed that this protein did not behave as if it were homogeneous. The authors concluded that the name "glycinin" for the different proteins from soy meal was not in accord with the data, since the protein preparations which they studied were mixtures of proteins.

Danielsson [9] studied soy globulin in the ultracentrifuge (M NaCl extract of the ground-up seeds, 70% saturated with ammonium sulfate, the precipitate dissolved in 0.2 M NaCl and dialyzed). In a buffer with ionic strength I = 0.3 and pH 7 the protein consisted of two components with sedimentation constants S = 13.1 and 8.0 Svedberg units.

Naismith [3] used the ultracentrifuge to study glycinin obtained according to Osborne and Campbell [10], Hartman and Cheng [11] (bringing an aqueous extract of soy bean meal to pH 4.5), and Briggs and Mann [8] (precipitating the protein from an aqueous extract of soy bean meal by allowing it to stand for several hours in the cold). The experiments were carried out at I = 0.5 and pH 7.8 (phosphate buffer). In no case was glycinin homogeneous. The sedimentation constants of the three basic components were 7, 11 and 16 Svedberg units. The author concluded that the name glycinin given in earlier work on soy bean protein corresponded to a mixture of proteins consisting at most of four components.

Wolf and Briggs [12] used the ultracentrifuge to study an aqueous extract of soy meal and found four well-defined protein fractions with S = 2, 7, 11 and 15 Svedberg units. The authors assumed that even these fractions were not homogeneous, but were mixtures of a number of individual proteins. For example, the fraction with S = 7 contained lipoxidase (S = 5.62), soyin (S = 6.4), and globulin with S = 7.97, and the fraction with S = 2 contained as its chief component a trypsin inhibitor, and a mixture of other proteins.

We can conclude from our data that the macromolecule of glycinin consists of identical submolecules which can associate reversibly, so that glycinin under different conditions of solution may change its molecular weight within wide limits. Thus, the degree of association is determined by the conditions of the surrounding medium, and so we can find varying amounts of the components which make up glycinin. Analogous data have been obtained in an orienting study by the method of electrophoresis.

Therefore we can say that the name glycinin corresponds to a globulin of soy cotyledons which consists of submolecules forming a stable hexamer (S_8-component) which can associate depending on the conditions in the medium.

The enzymatic function of glycinin is interesting in this connection [13]. Since in none of the conditions which we have studied have we found any admixture of other proteins, we can assume that the observed enzyme activity belongs to the protein itself. The role of soy globulin in sprouting seems interesting and important; it is evidently the source of the increasing enzyme activity in sprouting seeds. It is also interesting that the molecular weight of the glycinin submolecule (20,000) is close to the molecular weight of the "albumin" from soy cotyledons (16,000). Possibly the "albumin" is a product of the degradation of globulin.

SUMMARY

We can conclude that glycinin actually consists of submolecules which because of van der Waals forces form a stable hexamer (the S_8 - component), capable of further association because of predominating electrostatic forces.

The assumed mechanism for the reversible association of glycinin agrees well with the observed facts. The basic ideas of this mechanism are the following.

1) At pH below the isoelectric point there is a reversible equilibrium between the submolecules and the hexamer, which depends on the antagonism of the van der Waals and electrostatic forces and also on steric factors.

2) At pH above the isoelectric point van der Waals and electrostatic forces act in the same direction and permit the formation of oligomers of the stable S_8 - component.

3) Increase in ionic strength removes most of the electrostatic effect by screening the free charges with opposite ions.

Received September 12, 1957

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*** Russian translation.

PROPERTIES OF SPERMOSIN, THE CONTRACTILE PROTEIN OF SPERMATOZOA

S. A. Burnasheva

A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR, Moscow

A considerable number of papers have been published over the past few years showing that the biochemical processes involved in muscle contraction are of general biological significance, applying to a variety of forms of motor activity, such as contraction, cell division, and others.

The capability of performing mechanical work is one of the basic properties of living matter. The evolutionary development of this property has attained its highest level in muscle tissue. The basic features of muscle tissue are probably also present in less specialized tissues and in primative cells, including spermatozoa, which are able to convert chemical energy into mechanical work.

The results of electron-microscopic examination of spermatozoa [1,2] are of interest, in the light of the theory of muscular contraction, which is regarded as being the result of a reaction between low-molecular crystalloid metabolites with high-molecular protein structures, of which the contractile apparatus of muscle cells is composed. It has been shown that the contractile apparatus of the tail of spermatozoa resembles that of muscles, and consists of fibrils surrounded by the spiral thread. It has, on the other hand, been shown that adenosinetriphosphate (ATP) plays an important part in the motor function of spermatozoa, and that ATP-ase activity is localized in their caudal portion [5 – 7], i.e., in the contractile mechanism of the spermatozoon. It has been shown [7] that the protein spermosin, isolated earlier from bull, ram, and boar spermatozoa, and possessing ATP-ase activity, is also located in the tail, and is therefore presumably an integral part of the motor apparatus of the spermatozoon. It may, on the above evidence, be concluded that the movements of the tail of spermatozoa are achieved by means of rhythmic structural changes taking place in the contractile protein, due to its reaction with the energy-rich substance adenosinetriphosphate.

In the present paper we report the results of further study of the properties of spermosin. We thought it would be of interest to examine the mechanical-chemical properties of spermosin, i.e., the nature of the reaction of spermosin, as an enzyme, with its substrate ATP. In particular, we wished to elucidate whether spermosin, in acting enzymatically on ATP, would at the same time itself undergo any changes due to the action of ATP, such as are encountered with muscle proteins. We hoped first of all to find out whether spermosin resembles myosin in being a single protein, or whether it is a protein complex of the type of actomyosin. Such studies would contribute to the elucidation of the question as to whether one single protein, known to be an integral part of the organism, suffices for the achievement of the elementary act of contraction, in conjunction with ATP as a source of energy, or whether a complex of two proteins, similar to actomyosin, is involved.

EXPERIMENTAL

Preparation of spermosin from spermatozoa

The experimental material consisted of bull sperm, from which the spermatozoa were separated by centrifugation, followed by 2 or 3 washes with 0.85% NaCl (0.14 M). Spermosin was isolated from a homogenate of disintegrated spermatozoa containing both the heads and the tails, as it has been shown that there is no difference between the spermosin isolated from whole organisms or from the tail portions alone. In our earlier experiments the homogenates were prepared by grinding the organisms under liquid air. Although this procedure gave high yields of protein, we found that its ATP-ase activity had been very considerably lowered (by 40 – 70%).

For this reason, in our subsequent experiments we used a glass homogenizer at 0°, and in some of the experiments a Waring Blendor type of homogenizer, working at 15,000 rpm. The homogenates were extracted with various salt solutions, used for the isolation of myosin and of actomyosin. The following salt solutions were used: 1) Bailey's solution, 0.5 M KCl + 0.03 M $NaHCO_3$, pH 8.5; 2) Weber-Meyer solution, 0.6 M KCl + 0.04 M $NaHCO_3$ + 0.01 M Na_2CO_3, pH 9 [8]; 3) Hasselbach and Schneider's solution, 0.47 M KCl + 0.01 M pyrophosphate + 0.1 M phosphate buffer, pH 6.4 [9]; 4) 7% lithium chloride solution, pH 7 [10]. The proportions of centrifugate to supernate were taken as 1 : 5 and 1 : 10. The duration of extraction varied. For extraction of the myosin-like protein we took a time of 30 min, while for extraction of actomyosin-like protein the duration was from 24 to 48 hr. The proteins were precipitated from the salt solution extracts by lowering the salt concentration to 0.025 – 0.05 M, at pH 5.5 – 6, or by salting out with ammonium sulfate at 40 – 50% saturation.

We determined the ATP-ase activity of the initial homogenate, of the extracts, and of the proteins isolated; the activity was expressed as μg of phosphorus split off from ATP per mg of protein N in 10 min at 37°. Inorganic phosphorus was determined according to the method of Fiske and Subbarow, with the difference that instead of eikonogen we used ascorbic acid, as suggested by Lowry and Lopez [11]. Nitrogen was determined colorimetrically, by Nesslerization, after digestion.

EXPERIMENTAL RESULTS

Determination of yield of protein and of ATP-ase activity of extracts and of spermosin fractions

The results of the experiments designed to ascertain the yields of protein and of ATP-ase activity of the extracts and of the spermosin fractions, obtained using various solutions of high ionic strength, are presented in Table 1.

TABLE 1

Yields of Protein and of ATP-ase Activity in Extracts and in Spermosin Fractions

Nitrogen content and over-all activity found in salt solution extracts and in spermosin fractions, expressed as percentages of the corresponding values for homogenates

Expt. No.	Solution used for extraction	Duration of extraction, hr	Extract		Spermosin fraction	
			N	activity	N	activity
1	0.5 *M* KCl – 0.03 *M* $NaHCO_3$, pH 8.5	0.5	14.5	25.0	5.7	8.0
2	The same	0.5	14.4	34.0	3.6	17.0
3	"	2.5	33.0	60.0	19.0	55.7
4	"	24.0	25.0	92.0	2.5	42.0
5	"	24.0	27.0	76.0	11.0	24.0
6	0.6 *M* KCl + 0.04 *M* $NaHCO_3$ + + 0.01 *M* Na_2CO_3, pH 9.2	20.0	42.2	58.0	18.4	34.0
7	The same	24.0	30.0	78.0	7.0	26.0
8	7% LiCl, pH 6.2	24.0	49.0	65.0	17.7	48.6
9	0.47 M KCl + 0.01 M pyrophosphate + + 0.1 M phosphate buffer, pH 6.4	24.0	11.0	81.0	8.2	20.0

Note: Spermosin was precipitated by dialysis in experiments 1, 2, 4 and 5, and by salting out with ammonium sulfate to 50% saturation in the remaining experiments.

As is evident from Table 1, 2.5 – 18.4% of the nitrogen, and 8 – 55% of the enzymic activity of the homogenates are to be found in the spermosin fractions. The yields of protein and of enzyme activity rise with increasing duration of extraction. The different solutions tested are of almost identical extractive capacity.

2. Effects of Mg, Ca, and ATP ions on the relative viscosity of spermosin

We examined the effects of magnesium, calcium, and ATP ions on the relative viscosity of spermosin, prepared by short (30 min) and prolonged extraction (24 – 48 hr) with the above-specified salt solutions of high ionic strength, with the object of ascertaining whether the protein extracted from spermatozoa had con-

tractile properties similar to those of myosin or of actomyosin. It is known that prolonged extraction of muscle removes actomyosin, characterized by its high viscosity. When treated with ATP, actomyosin dissociates to give its constituent proteins, and this is associated with a fall in viscosity.

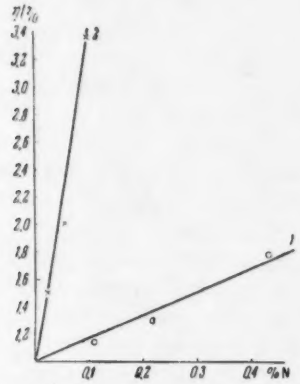

Fig. 1. Dependence of relative viscosity of spermosin on its concentration. 1) Relative viscosity of spermosin; 2) relative viscosity of myosin.

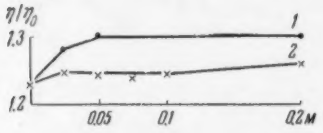

Fig. 2. Effect of calcium and magnesium ions on the relative viscosity of spermosin solutions. 1) Spermosin + Ca^{++}; 2) spermosin + Mg^{++}.

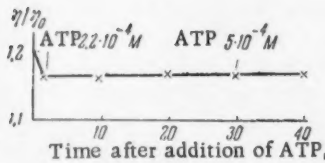

Fig. 3. Effect of ATP on the relative viscosity of spermosin solution.

The viscosity of solutions of spermosin in 0.4 – 0.5 M KCl at pH 7 was measured, using an Ostwald viscosimeter. The temperature was 18 – 20°.

Our measurements showed that the relative viscosity of spermosin was low, irrespective of the nature of the solvent system or of the duration of extraction, and that the same values were found in all cases.

Figure 1 illustrates the relation between relative viscosity of spermosin and its concentration. Analogous data for myosin are presented, for purposes of comparison.

Magnesium and calcium ions had no significant effect, at concentrations of 0.0001 – 0.01 M, on the relative viscosity of spermosin, prepared by short or prolonged extraction of spermatozoa (Fig. 2).

Addition of ATP, at concentrations of $2.2 - 5 \times 10^{-4}$ M, as well as at lower concentrations of the order of $10^{-5} - 10^{-6}$ M, and both in the presence and the absence of Mg and Ca ions, had no effect on the relative viscosity of spermosin prepared by prolonged extraction, as would have been found with actomyosin (Fig. 3). It may be concluded from these experiments that our extracts do not contain a protein of similar properties to those of actomyosin.

Attempts at reprecipitation of spermosin, with the object of separating it from possible admixtures of protein of the type of actin, showed that no significant amount of any such protein could be demonstrated; it should precipitate out at pH 10, from 0.05 M KCl solutions. Under such conditions, practically all the protein remained in the supernate, from which all the protein present in the initial spermosin fraction was precipitated when the KCl concentration was lowered to 0.025 M, and the pH to 5.8 – 6.2. The entire ATP-ase activity was found in this precipitate. The same results were obtained when the reprecipitations were repeated.

3. Attempts at isolation of an actin-like protein from spermatozoa

Attempts were made to isolate an actin-like protein from spermatozoa, in order to obtain direct evidence of the presence or absence of a protein of this type.

Straub's procedure [12] was applied, with certain modifications, to the isolation of actin from spermatozoa. Spermatozoa were treated with acetone, and dried, and the powder was extracted with 20 volumes of water for 30 min. Taking 200 mg of powder, we found that 0.54 mg passed into the extract; calculated as nitrogen, this would mean that 0.2% of the total nitrogen content of the spermatozoa passes into the "actin" fraction. Prolongation of the time of extraction did not increase the

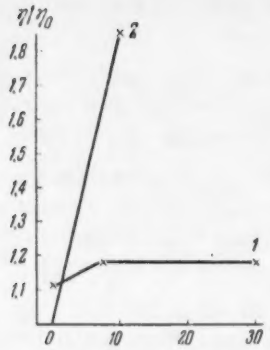

Fig. 4. Relative viscosity of spermatozoidal "actin" solutions and of muscle actin solutions, in the presence of Mg ions. 1) Spermatozoidal "actin" + Mg^{++} (10^{-3} M); 2) muscle actin + Mg^{++}.

amount of water-soluble extractives. The relative viscosity of the extract, containing 0.4 mg of protein per ml, is expressed by $\eta / \eta_0 = 1.12$. Addition of Mg^{++}, to concentrations of 10^{-3} M or of $10^{-2} - 10^{-5}$ M, did not raise the relative viscosity of the solutions, whereas addition of magnesium ions to muscle actin solutions causes an abrupt rise in viscosity (Fig. 4). The viscosity of the extracts was not affected by varying the magnesium concentration. It may be concluded that the protein extracted from spermatozoa by a procedure analogous to that used for extraction of actin is not activated by magnesium ions, as is muscle actin. Addition of spermatozoidal "actin" to myosin solutions, in the proportion of 5 : 1, or in other proportions, was not followed by a rise in the viscosity of the mixture, showing that a complex of the type of actomyosin was not formed. Addition of the spermatozoidal "actin" to spermosin solutions did not cause any rise in relative viscosity, in the presence or the absence of magnesium ions.

It may be concluded from these experiments that spermatozoa do not contain a protein analogous to the actin of muscle, and that the contractile protein of spermatozoa — spermosin — resembles myosin rather than actomyosin.

4. Formation of a complex ("actospermosin") from spermosin and muscle actin

Additional evidence of the similarity between spermosin and myosin was afforded by experiments on the formation of protein complexes of spermosin with muscle actin. If spermosin does in fact resemble muscle myosin in many respects, it could be expected that its addition to F-actin solutions should be followed by a rise in viscosity, due to complex formation, and that addition of ATP to the mixture should cause dissociation of the complex, with lowering of viscosity.

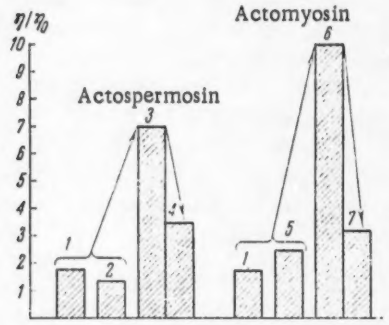

Fig. 5. Formation of the protein complex actospermosin from spermosin and muscle actin. Relative viscosity: 1) F-actin; 2) spermosin; 3) spermosin + F-actin (12 : 1); 4) spermosin + F-actin + ATP (4.23×10^{-4} M); 5) myosin; 6) myosin + F-actin (2 : 1); 7) myosin + F-actin + ATP (4.23×10^{-4} M).

We found that when spermosin was added to muscle myosin, in the proportions of 2 : 1, 4 : 1, 6 : 1, 8 : 1, and 12 : 1, there was a considerable rise in the relative viscosity. The viscosity of the actospermosin complex formed is more than double the sum of the viscosities of its components (Fig. 5, column 3). After addition of ATP, at a concentration of 4.23×10^{-4} M, the relative viscosity falls to the value of the sum of that of the components (column 4). For purposes of comparison, the corresponding changes occurring in the relative viscosities of mixtures of muscle actin and myosin are shown in Fig. 5. Formation of actomyosin is associated with a considerable rise in relative viscosity, as compared with that of myosin alone (column 6). Addition of ATP is followed by a fall in viscosity to the level of myosin alone (column 7).

Thus, these experiments show that spermosin resembles myosin in its ability to form a complex with muscle actin. Addition of ATP causes dissociation of this complex, as is shown by the resulting fall in relative viscosity. The results of these experiments thus confirm our view that spermosin resembles myosin.

In conjunction with the results of electron microscopic examination of spermatozoa [1,2], which show that the body of a spermatozoon consists of a bundle of fibrils surrounded by the spiral thread, each fibril being capable of executing rhythmic oscillatory movements, our results suggest that contraction of the body may be achieved by means of rhythmic structural changes in the contractile protein of the tail; this protein represents a unicomponent system, similar to muscle myosin, reacting with the high-energy compound adenosinetriphosphate. We have not, however, been able to obtain direct experimental evidence of reaction of the high-energy substance ATP with spermosin. Threads drawn from salt solution extracts of the tail parts of spermatozoa of the marine invertebrate Asterias chlatopteris [13] did not contract when treated with ATP. Addition of ATP to the protein isolated from these extracts led to its superprecipitation. However, Hofman-Berling [14], using a model system consisting of spermatozoidal tails extracted with aqueous glycerol, was able to show that they contracted when treated with 5×10^{-6} M ATP.

SUMMARY

Various procedures have been applied to the isolation of spermosin, the contractile protein of bull spermatozoa. From 2.5 to 18% of the total nitrogen content of spermatozoon homogenates was found in the spermosin fraction, and from 8 to 55% of their enzymatic (ATP-ase) activity.

Spermosin has many features in common with myosin, the contractile protein of muscle. These are: a) similarly to myosin, it exhibits ATP-ase activity, b) like myosin, it forms a complex with muscle actin, c) both actomyosin and actospermosin undergo dissociation when treated with ATP, as is shown by the fall in viscosity.

No protein fraction resembling muscle actin could be isolated from the spermatozoa. It is probable that the contractile protein of spermatozoa is a unicomponent system, analogous to myosin, rather than a bicomponent system such as actomyosin.

Received September 16, 1957

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** Original Russian pagination. See C. B. Translation.

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THE ADSORPTION OF STRONTIUM ON HYDROXYAPATITE CRYSTALS

M. D. Morachevskaia, V. S. Zlobin and B. V. Ptitsyn

Technological Institute of the Food Industry, Ministry of Higher Education, USSR, Leningrad

The problem of prevention of entry of radioelements into the human organism and their removal if they have entered is now of great significance.

Especially important in this respect are the strontium isotopes Sr^{89} and Sr^{90} with half lives of 54.5 days and 19.9 years. Uptake of strontium by the organism is due to its chemical similarity to calcium. Absorption of strontium from the intestine occurs in the same way as for calcium. Of the total strontium which enters the stomach, from 5 to 60% is absorbed into the blood, depending on conditions [1]. Norman, MacDonald, and Ralph [2,3] showed that absorbed strontium actively suppresses calcification of the growing bone even with favorable dietary ratios of calcium and phosphorus and adequate amounts of vitamin D and A. Strontium is deposited in bone in two phases. In the first, very rapid phase, strontium is transferred from the blood onto the adsorbing surface of the inorganic base of bone and undergoes ion change with calcium, becoming bound by the protein of the periosteum. The second phase is found on long exposure to sufficient amounts of strontium. In this case it is included in the crystal lattice of bone. Besides strontium, a whole series of radioelements, zirconium, yttrium, barium, radium, lanthanum, actinium, thorium, uranium, neptunium, plutonium, and others, are taken up by bone tissue. Such an uptake of radioelements forces us to recognize the great adsorptive properties of the crystalline salts which make up the inorganic phase of bone.

It is known that the limits of permissible dose for a single injection of radiostrontium as a soluble compound is, for a 70-kg man, 5.5 mC. The importance of not exposing the organism even to a minimal dose of radiostrontium in the internal environment is obvious.

According to Remy [5], the mineral phase of bone consists of hydroxyapatite of the composition $3Ca_3(PO_4)_2 \cdot Ca(OH)_2$, a white, finely crystalline substance, difficultly soluble in water, but soluble in acids.

Our work is designed to study the adsorptive properties of hydroxyapatite with respect to strontium and to find a way of preventing the penetration of radiostrontium into the bone, when it is taken into the human organism in the diet.

EXPERIMENTAL

We prepared three samples of calcium phosphate which contained different amounts of calcium and phosphorus. Form 2 was prepared as follows. A boiling solution which contained 118 g $Ca(NO_3)_2 \cdot 4H_2O$, 27 g NH_4Cl, 50 ml of 20% NH_3, and 0.5 liters of water was treated slowly with stirring with 2 liters of hot 0.18 M solution of Na_2HPO_4. The solution with the precipitate stood for a day, then the clear solution was poured off, the precipitate was washed with hot water, at first by decantation and then on a Buchner funnel to a negative reaction for chloride ion. The precipitate was dried to constant weight at 110-120° and ground in a mortar.

Forms 1 and 3 were prepared in the same way; the difference was only in the amount of ammonia added and the order of mixing the solutions.

The amount of calcium and phosphorus in the preparations was determined after first adsorbing the calcium ions from the solution on a cationite resin SBSR [5]. Phosphorus was determined as magnesium pyrophosphate, calcium, by precipitation as the oxalate and weighing as the sulfate.

The results of the analyses of the three preparations of calcium phosphate are given in Table 1.

TABLE 1

Results of Analyses of Different Forms of Calcium Phosphate

Preparation no.	Mole ratio of Ca/P in the preparations, found experimentally	Assumed composition of precipitate
1	0.96	$CaHPO_4$
2	1.61	$3\,Ca_3(PO_4)_2 \cdot Ca(OH)_2$
3	2.15	$3\,Ca_3(PO_4)_3 \cdot 4Ca(OH)_2$

Form 2 (Ca/P = 1.6) is close to the composition of hydroxyapatite, $3Ca_3(PO_4)_2 \cdot Ca(OH)_2$, in which the mole ratio of Ca/P is 1.67.

We used the isotope Sr^{89} as the indicator in this work. Our preparation of Sr^{89} contained several percent of Sr^{90} as an impurity. Disintegration of Sr^{89} leads to non-radioactive Y^{89} which does not change the study of the adsorption of strontium by the radioactivity of the latter. As a result of the disintegration of Sr^{90}, radioactive Y^{90} is formed, in which the maximum energy of the β-rays is greater than in strontium. As a result, the measurement of the amount of strontium adsorbed by measuring the distribution of activity between solution and solid phase may be altered, so that the results will differ under different conditions of measuring the activity. For this reason, before studying the adsorption of strontium on hydroxyapatite, we separated the yttrium which had accumulated in the strontium. The starting solution of radiostrontium which contained about 1 mC Sr^{89} per ml (the salt was 0.1 mg/mC) was diluted 1000 times with water. To 5 ml of this solution was added 0.5 ml of 1.5 M HNO_3 and 10 mg yttrium nitrate. The solution was heated to boiling and treated with a 25% solution of ammonia until the odor appeared. The solution was kept with the precipitate for 5 minutes and then filtered on a Buchner funnel. Precipitation of yttrium hydroxide was carried out twice. It was shown experimentally that under these conditions the radioyttrium was removed almost completely and the resulting solution could be used for several hours without noticeable accumulation of yttrium.

TABLE 2

Relation of Amount of Adsorbed Strontium (in %) to Composition of Calcium Phosphate and Concentration of Strontium in Solution

Preparation no.	Concentration of $Sr(NO_3)_2$, M	
	$2.5 \cdot 10^{-7}$	$5 \cdot 10^{-5}$
1	27.9	23.1
2	21.6	21.1
3	17.3	13.0

Strontium solutions of different concentrations were prepared by diluting a 1 M solution of $Sr(NO_3)_2$, with the addition of a small amount of a solution of $Sr(NO_3)_2$ which contained radiostrontium. The concentration of active strontium was slight compared to the inactive strontium.

It was shown that adsorption of strontium by the hydroxyapatite powder proceeded rapidly and the amount of adsorbed strontium on form 2 did not change during a time interval from 5 min to 8 hours. In later work the amount of adsorbed strontium was determined after five-minute shaking of the solution with powdered calcium phosphate.

In each experiment 10 ml of solution was added to a sample of 0.5 g of calcium phosphate, the mixture was mixed energetically for 5 min, and then the powdered calcium phosphate was filtered on a Buchner funnel. The powder was washed with 10 ml of water and the filtrate was diluted to 20 ml; the residue was dissolved in 10 ml of N HNO_3. From this solution 0.2 ml was transferred to a watch glass and evaporated; the activity of the dry residue was measured. The activity was measured with a β-tube counter. Each experiment on adsorption was repeated twice; for the measurement of activity two samples were taken from each solution and the activity of each sample was also measured twice.

The first series of experiments was run to study the effect of the composition of the calcium phosphate and the concentration of strontium in solution on the amount of adsorption of strontium. Adsorption was carried out in a hydrochloric acid solution of $Sr(NO_3)_2$ at concentrations of $2.5 \cdot 10^{-7}$ M and $5 \cdot 10^{-5}$ M and 0.03 M HCl, which corresponded to the acidity of gastric juice (pH $\simeq$ 1.5).

The results of this series are given in Table 2.

As Table 2 shows, the value of strontium adsorption changes slightly with change in composition of calcium phosphate; it increases with decreased ratio of Ca/P. Later all the experiments were carried out with preparation 2, which was analogous in composition to hydroxyapatite, as we mentioned earlier.

The percent adsorption of strontium by calcium phosphate increases slightly with a decrease in concentration of strontium in solution by 200 times. The amount of adsorption of strontium by hydroxyapatite is not great. To increase it, the solution was neutralized by sodium bicarbonate (0.126 g $NaHCO_3$ in 10 ml of the 0.03 M HCl solution, final pH ~ 8).

The following data were obtained:

Molarity of strontium nitrate solution	Sr adsorption in %
$5 \cdot 10^{-3}$	47
$5 \cdot 10^{-4}$	87
$5 \cdot 10^{-5}$	99

Thus, when the solution was neutralized the adsorption of strontium by hydroxyapatite was sharply increased. However, when the hydroxyapatite is placed in the stomach to remove the strontium adsorbed on it, neutralization of the gastric juice by bicarbonate is not possible. After suppression of the secretion and neutralization of the acidity of gastric juice there is a second wave of hypersecretion, which can lead to partial desorption of the strontium. Also, sodium bicarbonate increases the reserve alkalinity of the blood which increases the adsorption on the bone of strontium which has already reached the blood. Therefore, instead of sodium bicarbonate we studied disodium phosphate which neutralizes gastric juice, suppresses its secretion for a long time, and does not cause a secondary wave of hypersecretion. Uptake of excess disodium phosphate in the blood does not cause a sharp increase in reserve alkali of the blood.

Adsorption of strontium by hydroxyapatite was carried out from solutions neutralized by disodium phosphate (3.3 ml 0.18 M Na_2HPO_4 in 10 ml $Sr(NO_3)_2$ solution with a HCl concentration of 0.03 M).

The following results were obtained:

Molarity of solution	Adsorption of strontium in %
$Sr(NO_3)_2$	90*
$5 \cdot 10^{-3}$	98
$5 \cdot 10^{-5}$	98
$5 \cdot 10^{-7}$	

To show that at small concentrations of strontium in solution the Na_2HPO_4 does not cause its precipitation, we ran an experiment without adding hydroxyapatite. To 10 ml of a $5 \cdot 10^{-7}$ M solution of strontium nitrate ($M_{HCl} = 0.03$) we added 3.3 ml of 0.18 M Na_2HPO_4, shook for 5 min and filtered. The amount of strontium retained by the filter was about 5%. Thus at small strontium concentrations in the solution its precipitation as the phosphate does not occur, but instead adsorption on the crystals of hydroxyapatite. At higher strontium concentrations, its precipitation does occur. Thus, when to 10 ml of $5 \cdot 10^{-3}$ M strontium nitrate solution ($M_{HCl} = 0.03$) was added 0.252 g of sodium bicarbonate, about 88% of the strontium precipitated, and when 3.3 ml of 0.18 M Na_2HPO_4 was added, about 63% of the strontium precipitated.

To determine the effect of the order of mixing the components we ran an experiment in which Na_2HPO_4 was added 5 min after the addition of strontium nitrate to the sample of hydroxyapatite (in the experiments described earlier the solutions of $Sr(NO_3)_2$ and Na_2HPO_4 were first mixed and the resulting solution was added to the hydroxyapatite powder). The amount of adsorbed strontium at a starting concentration of $5 \cdot 10^{-7}$ M was 95%. Thus the order of adding Na_2HPO_4 is not significant.

We ran experiments on the adsorption of strontium from filtered human gastric juice. To the gastric juice was added a small amount of $Sr^*(NO_3)_2$ solution; 10 ml of gastric juice were shaken with 0.5 g of hydroxyapatite,

*As in original.

and then 0.084 g Na_2HPO_4 was added (which corresponded to 3.3 ml of 0.18 M solution).

The following results were obtained:

Molarity of $Sr(NO_3)_2$ solution	Adsorption of Sr, %
$5 \cdot 10^{-5}$	96
$5 \cdot 10^{-7}$	98

From these data it follows that adsorption of strontium by hydroxyapatite from gastric juice does not differ from its adsorption from water solution.

SUMMARY

We have studied the adsorption of strontium (using Sr^{89}) on crystals of hydroxyapatite from water solution and gastric juice.

Adsorption of strontium by hydroxyapatite from acid solution (pH-1.5) at strontium concentrations in the solution of $2.5 \cdot 10^{-7}$ to $5 \cdot 10^{-5}$ M is small. When sodium bicarbonate or disodium phosphate are added to the solution, strontium is removed from the solution almost completely (at higher strontium concentrations there is precipitation as phosphate or bicarbonate).

When radiostrontium enters the stomach we recommend for its removal the administration per os of a water suspension of 10-20 g of hydroxyapatite with addition of 1-2 g of Na_2HPO_4.

Received September 27, 1957

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THE COMPOSITION OF THE NUCLEAR NUCLEOPROTEINS OF SOME PLANTS

A. N. Belozerskii and S. O. Uryson

A. N. Bakh Institute of Biochemistry, Academy of Sciences, USSR, Moscow

At the present time there is much information which casts light on the chemical composition of the cell nuclei of animals and the relations of the separate components in them. As to the cell nuclei of plants, only the nuclei and nuclear nucleoproteins of cereal embryos [1-7] and the nuclear nucleoproteins of a few other plants [8] have been studied. Such a lack for plant biochemistry can be mainly explained by the difficulties connected with isolating the cell nuclei from plant cells and tissues. It is also apparent that for a study of nuclear nucleoproteins and the ratios of the various components in them it would be necessary to produce the isolated cell nuclei. With all these difficulties in setting up the study, some progress could be made in investigating these problems, as we have assumed, by studying the nuclear nucleoproteins isolated directly from plant tissues and cells used for this purpose.

In our work we have made a comparative study of the nuclear nucleoproteins from germinating sprouts of some bean plants, cedar nut embryos and bacteria – Proteus vulgaris.

EXPERIMENTAL

For the beans we used haricot and soy beans, and as a representative of the conifers, cedar nuts (Pinus sibirica Rupr.). In the first case the nuclear nucleoproteins were obtained from germinating sprouts and in the second from the embryos. These parts of the plants seemed most suitable for our purposes since they could be considered embryonic tissue. We also studied the bacteria, Proteus vulgaris.

The cedar nut embryos were separated from the endosperm by hand, and the bean sprouts were first finely ground and then fractions were collected by suspending the enriched fractions obtained by sifting through a sieve in a 3 : 1 mixture of CCl_4-toluol (in the experiments with soy beans a second suspension in the same mixture with a ratio of 3 : 2 was necessary). The resulting embryos and sprouts were defatted with ether, and ground to a fine meal which served as the starting material for obtaining the nucleoproteins.

The bacteria, Proteus vulgaris was grown on a meat-peptone agar for 30 hours at 30°, washed from the agar with water, the suspension was centrifuged, the bacterial residue was washed with water and frozen. For the extraction of the nucleoproteins, the frozen bacterial mass and also the embryo and sprout meal were carefully ground with powdered glass in the presence of the corresponding extraction medium at 2°.

The extraction of the nucleoproteins was carried out on the basis of the method of Mirsky [2]. A weight of the material (10 g of defatted cedar nut embryo meal, 20 g of haricot and soy bean sprout meal, and 5-10 g of frozen bacterial mass) were placed in a beaker with 10 times the amount of 0.14 M NaCl in 0.015 M sodium citrate. The mixture was carefully ground with glass powder at a low temperature and then shaken in a shaking machine for 20-30 min. Separation of the extract from the residue was carried out by centrifuging. Extraction with the 0.14 M NaCl-0.015 M sodium citrate solution was carried out 6-7 times with gradually decreasing volumes of the salt solution to full removal of substances which passed into this solvent. The "nucleoproteins" were precipitated from the weak salt solution by dropwise addition of a 10% solution of acetic acid. The precipitate was washed with weak acetic acid and then dried lyophilically.

The residual material after extraction by 0.14 NaCl was treated in the cold 4-6 times with M NaCl (and in the case of Proteus vulgaris, 10 or more times). The combined 1 M extracts were diluted 10-fold with water by dialysis in 10 times the volume of water.

Since in the case of the Proteus vulgaris only a very slight precipitate appeared after dilution of the 1 M NaCl extract with water and we had only a limited amount of bacterial mass at our disposal, the nucleoproteins were precipitated by addition to the salt solution of two volumes of alcohol (preparation 4). We also collected a small amount of nucleoprotein precipitated by water to determine its content of desoxyribonucleic acid (DNA).

At the end of the dialysis in the case of the cedar nuts a fibrous precipitate appeared which was once more reprecipitated from M NaCl. In the other case (haricot and soy beans) nucleoproteins with a structure were not formed, and only a small amount of precipitated amorphous nucleoproteins could be collected (preparation 2).

After precipitation from M NaCl of the nucleoproteins by dialysis we were able to isolate from the solutions one more fraction of nucleoproteins by adding two volumes of acetone to the solution (preparation 3).

TABLE 1

Content of Nitrogen, Phosphorus, and Nucleic Acids in the Extracted Preparations of Nucleoproteins

In % dry weight

Preparation studied	Substance determined	Germinating haricot sprouts	Germinating soy sprouts	Cedar nut embryos	*Pr. vulgaris*
1. Nucleoproteins extracted by 0.14 M NaCl	Total nitrogen	13.06	11.16	12.97	12.92
	Total phosphorus	0.94	0.98	1.85	1.16
	Total nucleic acids	7.81	7.63	4.66	11.94
	DNA	0	0	0	0
	RNA	7.81	7.63	4.66	11.94
2 Nucleoproteins extracted by M NaCl and precipitated by water	Total nitrogen	13.52	10.37	13.05	—
	Total phosphorus	1.15	1.48	2.54	—
	Total nucleic acids	10.98	8.48	25.91	—
	DNA	5.28	3.43	21.57	6.63
	RNA	5.70	5.05	4.34	—
3. Nucleoproteins extracted by M NaCl and precipitated by acetone	Total nitrogen	13.84	13.88	14.07	—
	Total phosphorus	0.25	1.08	0.97	—
	Total nucleic acids	1.08	8.39	2.71	—
	DNA	0	0	0.90	—
	RNA	1.08	8.39	1.81	—
4. Nucleoproteins extracted by M NaCl and precipitated by alcohol	Total nitrogen	—	—	—	13.14
	Total phosphorus	—	—	—	2.11
	DNA	—	—	—	9.68
5. Nucleoproteins extracted by 0.05 M NaOH	Total nitrogen	12.78	11.34	13.69	—
	Total phosphorus	1.25	1.15	0.69	0.32
	Total nucleic acids	9.76	7.63	5.09	—
	DNA	trace	trace	3.64	0
	RNA	9.76	7.63	1.45	—

The residue insoluble in M NaCl was extracted 2-4 times with 0.05 M NaOH, after which the residue was discarded. The combined alkaline extracts were treated in exactly the same way as in obtaining the nucleoproteins extracted by 0.14 M NaCl (preparation 5). All our preparations were dried lyophilically.

In each of the preparations we determined total nitrogen by the micro-Kjeldahl method, phosphorus by the Embden method, total nucleic acids spectrophotometrically [9], DNA according to Dische, ribonucleic acid (RNA) calculated by difference between total nucleic acids and DNA.

In Table 1 we give the analytical data which characterize the preparations. All the data are expressed in percent dry weight of the preparations.

The data of Table 1 show that nucleoprotein preparations extracted by 0.14 M NaCl in all cases are characterized by the complete absence of DNA and contain only RNA.

Thus we can assume that this fraction corresponds to the cytoplasmic components and does not contain nuclear nucleoproteins. The latter, judged by the DNA content, are chiefly present in the nucleoprotein fraction extracted by M NaCl and precipitated by water. We also established the presence of nuclear nucleoproteins for cedar nuts and Pr. vulgaris in the fractions precipitated from the M NaCl extract by acetone and alcohol, and in the case of the cedar nuts also in the fraction extracted by 0.05 M NaOH. It should be mentioned that all the fractions were characterized by a greater or less quantity of RNA. The presence of appreciable amounts of RNA not only in the fraction extracted by 0.14 M NaCl, but also in the other fractions, indicates the existence in the plants which we studied of ribonucleoproteins which differed in their properties.

As already mentioned, the most typical fraction, from the viewpoint of nuclear nucleoproteins, was the fraction extracted by M NaCl and precipitated by water. Therefore, for further study of the nuclear nucleoprotein composition we used this fraction. In the case of Pr. vulgaris for comparison we also studied the fraction of nuclear nucleoproteins obtained by precipitating the M NaCl extract with alcohol.

TABLE 2

Characteristics of Protein Preparations Obtained from Nuclear Nucleoproteins and from the Bacterial Mass by Extraction with HCl

In % of dry weight

Object of investigation	Total nitrogen	Tryptophan	RNA
Haricot	14.06	1.38	1.67
Soy	13.38	1.57	1.74
Cedar Nuts	16.52	0.23	0.78
Proteus vulgaris			
Nuclear nucleoproteins	9.31	0.88	—
Whole bacterial mass	12.38	1.49	11.60

We are most interested in the question of the presence of histone-type proteins in these nuclear nucleoprotein preparations.

For this study the lyophilically dried nucleoproteins were extracted 4-5 times with 0.2 N HCl and the combined hydrochloric acid extracts were treated with ammonia to a pH of 10-11. Under these conditions we obtained a well-defined flocculent precipitate only from the extract of the cedar nut proteins which had a definite structure. In the other cases there was only an increased opalescence in the solution without precipitation of a protein. A precipitate was obtained in the case of haricot and soy beans only after adding two volumes of alcohol to the hydrochloric acid extract which had been brought to pH 10-11.

In these acid soluble protein preparations we determined the content of nitrogen, the tryptophan by the method of Furth in the modification of Zbarskii [10], the nucleic acids spectrophotometrically, and the DNA according to Dische; we thus showed that DNA was absent in all the preparations.

The results of the analyses are given in Table 2 and expressed in percent of dry weight of the preparations.

Table 2 shows that all of the studied preparations can be considered to belong to the class of proteins with a high nitrogen content. Of all these preparations apparently only the one from the cedar nut embryo nuclear nucleoproteins is a typical histone. This preparation not only has the very low content of tryptophan which is characteristic of histones, but it is precipitated at pH 10-11, that is, it has the isoelectric point characteristic of the precipitation of histones. All the other preparations are characterized by a relatively high tryptophan content, lower total nitrogen content, and are not precipitated from solution at pH 10-11.

Thus the preparations of acid-soluble proteins from haricot, soy, and Pr. vulgaris do not have the characteristic properties of histones.

For further characterization of our acid soluble protein preparations we used the histone reaction of Daly, Mirsky, and Ris [11].

TABLE 3

Production of the Color Reaction for Histones in Acid Soluble Protein Preparations

Name of preparation	Intensity of color in (E/mg protein)
Histone from thyroid gland	5.6
Histone from cedar nuts	5.6
Acid-soluble protein of haricots	1.2
Acid-soluble protein from soy	1.8

The reaction was carried out as follows: a sample of protein (3-5 g) was suspended in 2.5 ml of water, to the suspension was added an equal volume of a solution of 0.34 M $HgSO_4$ in 1.88 M H_2SO_4 and it was heated 15 min at 60°. Then the mixture was cooled, 1/20 its volume of 1% $NaNO_2$ was added, and it was again heated 10 min at 60°, the solution was centrifuged and the intensity of the color of the supernatant liquid was determined in the electrophotocolorimeter at 500 mμ.

Table 3 gives the results of this investigation expressed in values of intensity of color per 1 mg of protein.

TABLE 4

Characteristics of the Nucleoprotein Residue Insoluble in 0.2 N HCl

In % dry weight

Object of investigation	Nitrogen	Phosphorus	Total nucleic acids	DNA	RNA
Haricot	12.65	2.74	22.99	17.19	5.80
Soy	12.85	1.97	15.85	6.36	9.49
Cedar nuts	14.30	4.37	48.05	39.08	9.97

The data of Table 3 show that when the reaction of Daly, Mirsky, and Ris is carried out, only the acid soluble protein from cedar nuts reacts as a histone and in this respect is analogous to the typical histone from the calf thyroid. The acid-soluble proteins from the haricot and soy beans cannot be characterized as proteins of the histone type by the reaction of Daly, Mirsky, and Ris.

Table 4 gives the characteristics of the nucleoprotein residue insoluble in 0.2 N HCl from the M NaCl extract. All the analytical data are expressed in percent of dry weight of the preparation.

From comparison of the analytical data for the preparations of nuclear nucleoproteins before extraction with acid (Table 1) and after it (Table 4) we can gain some idea of the amount of protein extracted by acid and the ratio of DNA and different types of proteins in these preparations. The content of acid-soluble proteins in the haricot nuclear nucleoprotein is ~40% and for soy beans, ~30%; in spite of this, these proteins do not have the properties typical of histones. It follows that if part of their RNA enters the solution along with the protein, the DNA must remain in the insoluble residue. As to the nuclear nucleoprotein of the cedar nut which has a structure, its content of histone-type protein, removed by acid, is about 23% and there is a corresponding accumulation of DNA in the residue of almost 40%. According to these results, nuclear nucleoprotein from cedar nut embryos resembles the corresponding nuclear nucleoprotein from the embryo of wheat and some animals [5,6].

DISCUSSION OF RESULTS

Comparative study of the nuclear nucleoproteins of plants is of great interest, since the literature on this question is very scarce.

Mirsky and Pollister [2] described the isolation from plant tissues of nuclei and chromosomes with a definite nucleoprotein fraction composed of DNA, histone, and complex proteins, and characterized by a fibrous structure when precipitated by water. These structured nucleoproteins also had the ability of giving very viscous solutions in M NaCl. The authors showed [2] the presence of such structured nucleoproteins in wheat embryos and pneumococcus. Later in our laboratory we isolated a structured nucleoprotein from wheat embryo and studied it in detail [5,6]; in its structure and essential properties it resembled the corresponding nucleoproteins of animal tissues. Naturally, it was interesting to see how widely nucleoproteins of this type were

distributed in the plant kingdom. It was equally important to establish how widely distributed in plants were the alkaline proteins of the histone type, since up to now such histones have been characterized in a more or less detailed way only in the structured nucleoproteins of wheat embryo [5,6].

In the present work we planned to study the nuclear nucleoproteins of beans (haricot, soy) and conifers (cedar nuts) for plants, and bacteria, Proteus vulgaris. Typical structured nucleoproteins, analogous in composition and properties to nucleoproteins from wheat germ and animal tissues, were found only in the cedar nut embryos. This nucleoprotein gave viscous solutions in M NaCl, had a fibrous structure when precipitated by water, contained typical histones and, finally, was characterized by the same ratio of histone and DNA (about 1 : 1), as in the other structured nucleoproteins. In the nuclear nucleoproteins from haricot and soy beans and the bacteria, Proteus vulgaris, we could not isolate a fraction of structured nucleoproteins, nor any proteins of the histone type. Our failure in the case of the beans (haricot, soy) was apparently not because alkaline proteins of the histone type were uncharacteristic of these plants, but because the germinating sprouts have a relatively low DNA content. The experimental work with the nuclear nucleoproteins showed that there was a direct relation between histone content and DNA content and the formation of a structured nucleoprotein fraction was apparently possible only when the DNA polymers and histone occurred in the corresponding ratio. Hence we believe that at very low DNA concentrations and in the presence of considerable amounts of other protein fractions, conditions exist which prevent us from obtaining any sort of typical structured nucleoprotein fraction, and therefore also of histones when the usual methods are employed for this purpose.

Another explanation can be given in the case of Proteus vulgaris. This organism contains about 4% DNA in the dry bacterial mass. Even so, we could not obtain either a structured protein or histone from it, though we tried to isolate the latter directly from the bacterial mass. Thus we have much more reason to doubt that the nuclear nucleoprotein of Proteus vulgaris contains proteins of the histone type.

In conclusion we should mention that in the objects of our investigation RNA was present in greater or less amounts in all our protein fractions. This fact, in our opinion, indicates the existence of ribonucleoprotein complexes with differing properties.

SUMMARY

We have isolated and studied the nuclear nucleoproteins from germinating sprouts of haricot and soy beans, cedar nuts, and Proteus vulgaris.

Typical structured nucleoproteins and typical histones have been found only in the cedar nut embryos. We have not been able to isolate histones either from the nuclear nucleoproteins of Proteus vulgaris or by direct extraction of the bacterial mass.

Received October 3, 1957

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A STUDY OF THE LOW MOLECULAR WEIGHT PRODUCTS OF PEPSIN DIGESTION OF HEMOGLOBIN BY PAPER CHROMATOGRAPHY

V. V. Oppel'

Biochemical Laboratory, Chair of Physiology of Military Activities,

S. M. Kirov Military Medical Academy, Leningrad

In previous communications [1,2] we showed that after pepsin digestion of hemoglobin, the lowest molecular weight fraction which was not precipitated by 0.78 M CCl_3COOH (final concentration in the solution) was a mixture of polypeptides which differed in amino acid composition. The "average" number of amino acids in the molecule equalled four. Certain amino acids accumulated in this fraction (arginine, phenylalanine).

In the work of Rovery and Desnuelle [3,4] it was shown that the hydrolyzate from pepsin treatment of horse globin contained free phenylalanine and peptides which contained leucine, alanine, and valine combined with various other amino acids.

The problem of the present study was to try to analyze the mixture of products of the pepsin digestion of hemoglobin which were not precipitated by 0.78 M CCl_3COOH and which were called fraction F-3 in the previous papers.

METHODS

Pepsin hydrolysis of hemoglobin was carried out from 30 min to 3 hours at 37°. The hemoglobin and pepsin were the same as in the previous study. The ratio of pepsin N to hemoglobin N in most of the experiments was 1 : 755. Before the solutions of hemoglobin and pepsin in 0.1 N HCl were mixed, they were brought to the temperature of the thermostat.

In a series of experiments we first separated from the hydrolyzate a hemoglobin fraction which contained a mixture of peptides with an average length of 11-12 (0-1) to 7-8 (0-2) amino acid residues. The ratio of pepsin N and polypeptide N varied in these experiments between 1 : 471 and 1 : 591. After precipitating with 0.78 M CCl_3COOH, we obtained fractions F-4 and F-5 (soluble in trichloroacetic acid) which had about the same chain length (four amino acid residues) as fraction F-3. In these experiments digestion also lasted 1 hour.

The trichloroacetic acid filtrate was freed from acid by repeated shaking with ether and evaporation on the steam bath. The precipitate was dissolved in a mixture of 95% ethanol and 0.1 N HCl (2 : 1) and analyzed chromatographically.

For one-dimensional or two-dimensional descending chromatography we used the following solvents: 1. n-butanol – acetic acid – water (25 : 6 : 25), 2. n-butanol – ethanol – NH_3 – H_2O (20 : 60 : 7 : 13). "Rapid" chromatography paper of the Volodar factory was first treated with weak HCl, carefully washed with water and then dried. We used a 0.2% solution of ninhydrin in n-butanol to color the spots. The chromatogram, after being sprayed with ninhydrin, was placed in a drying oven at 90° for 10 min.

Table 1 gives the R_f values for the pure amino acids found on the two-dimensional chromatogram with solvents 1 and 2 (temperature of the chamber 16-19°, distance covered by the solvent front, ~ 20 cm).

When the temperature was raised to 25 - 27° the R_f values changed considerably, which we allowed for in identifying the amino acid spots. For example, R_f for leucine increased to 0.6/0.69, for alanine, to 0.33/0.44,

glutamic acid to 0.26/0.17, lysine to 0.14/0.25, glycine to 0.24/0.34*, etc.

We used two procedures for the analysis of the composition of the peptides obtained by digestion of hemoglobin. On a wide sheet of chromatographic paper we drew a series of continuous horizontal lines and then carried out one-dimensional chromatography with the solvent n-butanol–acetic acid. After drying the chromatogram in a stream of air, we cut off narrow strips from both sides and developed the polypeptide spots on them with 0.2% ninhydrin in n-butanol. From the uncolored part of the sheets we cut out zones which corresponded to the spots on the developed chromatogram in the form of narrow strips which were submitted to further treatment.

TABLE 1

Rf Values for Pure Amino Acids
Two dimensional chromatogram; solvents: 1. n-butanol–CH_3COOH–H_2O (25 : 6 : 25); 2. n-butanol – ethanol – NH_3 – H_2O (20 : 60 : 7 : 13). Temperature 16-19°, front at a distance of ~ 20 cm. Average of 3-4 determinations

Amino acid	Solvent		Amino acid	Solvent	
	1	2		1	2
Leucine	0.59	0.61	Threonine	0.18	0.38
Phenylalanine	0.51	0.57			
			Glutamic acid	0.17	0.12
Valine	0.47	0.51	Glycine	0.15	0.25
Methionine	0.33	0.51	Serine	0.12	0.28
Tyrosine	0.26	0.44	Asparagine	0.13	0.09
Proline	0.26	0.38	Arginine	0.07	0.20
Alanine	0.26	0.33	Lysine	0.06	0.16

Since this method, when used in the study of the products of pepsin digestion of corticotropin [5], was shown to be unsuitable in a number of cases, we used another procedure. We carried out two dimensional chromatography on several sheets simultaneously. The sheets were cut to the same dimensions with the same paper. Solvents: 1 and 2. At the end of the separation, one of the sheets was treated with ninhydrin to determine the "coordinates" of the spots for the peptides, and on the chromatograms which had not been treated with ninhydrin circles were drawn in the corresponding places. These circles were cut out and all similar spots were treated together.

The strips or circles were placed in a test tube and treated with 5-10 ml of a mixture of 95% ethanol and 0.1 N HCl (2 : 1); removal of the peptides from the paper lasted not less than a day. The eluate was evaporated and the slight residue was dissolved in 2 – 3 drops of concentrated HCl and kept in a sealed capillary tube for 24 hours in a drying oven at 120°. At the end of the hydrolysis the contents of the capillary were transferred to a porcelain crucible, taken up in 5-10 ml of ethanol and the solution evaporated on the water bath. A second addition of ethanol and its evaporation fully removed the HCl. The small residue was dissolved in 0.1-0.2 ml of a mixture of alcohol and 0.1 N HCl (2 : 1). This solution was chromatographed: 1) one-dimensionally with a solvent of n-butanol–CH_3COOH (as controls we used 14 amino acids), 2) two-dimensionally with solvents 1 and 2. The two forms of chromatogram supplemented each other in the identification of the amino acids.

EXPERIMENTAL RESULTS

Reproducibility of the Results

The question arises whether strictly determined peptides are always obtained by pepsin hydrolysis of hemoglobin. To test this, we ran six experiments with one-hour digestion of hemoglobin.

In a series of experiments with the one-dimensional chromatogram (n-butanol-CH_3COOH), 20 spots were observed, many of which had Rf values close to the Rf of one or another amino acid. In particular we observed a spot with Rf 0.50-0.52 which corresponds to the Rf for phenylalanine (Rf = 0.52). The fastest moving spot

* In the numerator of the fraction we give the Rf value in solvent 1, and in the denominator, in solvent 2. This system is used later.

(R_f = 0.70-0.73 in one half of the experiments and 0.64-0.67 in the other) was unevenly colored and finished at a point farthest distant from the place of initial movement, which was a clearly colored and sharply limited triangle. The proximal section of the spot was paler in color and passed into a "tail".

The results of the one-dimensional chromatogram were not very satisfactory because of the formation of the "tail". Therefore, we used two-dimensional chromatography (acid butanol was used in the second portion and gave a clearer picture).

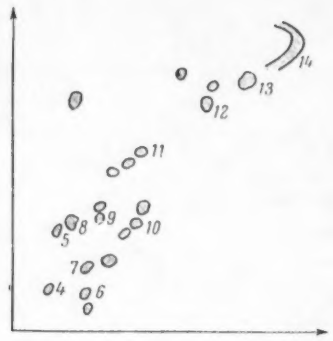

Fig. 1. Two-dimensional chromatogram of fraction F-3 of the one-hour pepsin hydrolyzate of hemoglobin. Solvents: vertically — n-butanol — ethanol — NH_3 — H_2O (20 : 60 : 7 : 13); horizontally — n-butanol — CH_3COOH-H_2O (25 : 6 : 25). Numbering of the spots corresponds to that in Table 5; spot 14 is complex (for explanation, see text); unnumbered spots are not characteristic (not found on the chromatograms in all the experiments).

Some of the spots on the chromatogram (Fig. 1) do not resemble in distribution the spots formed by any amino acid. Others have R_f values close to the R_f of amino acids. Some spots were found in all six experiments, or five of the six, the greater part of the spots were found less often. Some spots appeared in a single experiment.

The data of Table 2 show that R_f values for many spots are close to each other. In particular, nine spots have R_f from 0.02 to 0.15. In the case of the one-dimensional chromatogram, with the mixture of butanol and acetic acid many of the spots would be combined into one. Some rapidly moving spots which appear in the one-dimensional chromatogram with a "tail" are not found in the two-dimensional chromatogram. The original "triangle" which is obtained in the one-dimensional chromatogram is also found in the two-dimensional one, but it is greatly extended in both directions of the moving liquids. It is quite clear that this spot is complex (Fig. 1, spot 14).

A spot is observed on the chromatogram which corresponds to that of phenylalanine (R_f = 0.53/0.59). This spot is found in more than half the experiments (4).

Seven spots found in all, or five of the six, experiments must be considered as constant products of the pepsin hydrolysis of hemoglobin. If we consider the 11 spots each found in only one experiment (R_f for these spots 0.02/0.51; 0.05/0.84; 0.12/0.53; 0.16/0.34; 0.16/0.57; 0.33/0.22; 0.52/0.89; 0.38/0.51; 0.63/0.77; 0.78/0.51; 0.84/0.61) as accidental, there remain 20 spots found in two to four experiments which are evidently not accidental. Most probably they are not found on some chromatograms because of the relatively small concentration of the corresponding peptides.

In Table 3 appear the R_f values for the peptide spots obtained in different variations of the experimental conditions. Digestion of hemoglobin was carried out for 1 hour, and for 2-3 hours, or digestion was carried out with the products of pepsin hydrolysis which contained an average of 12 (Fraction O-1) or 8 (Fraction O-2) amino acid residues. The average size of the particles in the low molecular weight products of digestion of these fractions (Fractions F-4 and F-5) did not differ from the size of the peptides in Fraction F-3, which was obtained by hydrolyzing the whole hemoglobin [2].

Table 3 shows that, basically, when the conditions of digestion are varied, peptide spots appear on the chromatogram which have the same R_f values as in the experiments with one-hour digestion of hemoglobin. Among these is also found the spot with R_f characteristic for phenylalanine (0.53/0.59; 0.51/0.53; 0.53/0.54; 0.53/0.56). Some variation was found in the digestion of Fraction 0-1 (Fraction F-4). In this case, we noted the appearance of spots (with R_f : 0.06/0.36; 0.09/0.25; 0.63/0.55; 0.71/0.75) which were not found on other chromatograms. Since these spots were often found on chromatograms of Fraction F-4 (three or four experiments

TABLE 2

R_f of Peptides Obtained by One-Hour Hydrolysis of Hemoglobin by Pepsin

Two-dimensional chromatography; first solvent n-butanol – ethanol – NH_3 - H_2O (20 : 60 : 7 : 13), (2), second – n-butanol – CH_3COOH-H_2O (25 : 6 : 25) (1). Front at distance of 16-19 cm; temperature 17-20°, data of six experiments

Solvent		No. of expts. in which spots were found	Solvent		No. of expts. in which spots were found	Solvent		No. of experiments in which spots were found
1	2		1	2		1	2	
0.09(0.08—0.10)	0.18(0.17—0.19)	5	0.04(0.02—0.06)	0.10(0.08—0.12)	3	0.04(0.03—0.05)	0.18(0.17—0.19)	2
0.14(0.11—0.18)	0.19(0.17—0.21)	6	0.12(0.10—0.15)	0.46(0.41—0.48)	3	0.08(0.08—0.08)	0.64(0.63—0.65)	2
0.22(0.20—0.25)	0.14(0.10—0.16)	6	0.12(0.10—0.14)	0.31(0.29—0.33)	3	0.15(0.15—0.15)	0.10(0.10—0.10)	2
0.21(0.16—0.28)	0.32(0.30—0.34)	5	0.12(0.11—0.13)	0.12(0.10—0.16)	3	0.25(0.23—0.27)	0.57(0.55—0.58)	2
0.24(0.21—0.26)	0.35(0.33—0.38)	5	0.13(0.11—0.17)	0.74(0.70—0.77)	3	0.29(0.28—0.31)	0.75(0.73—0.76)	2
0.33(0.31—0.36)	0.32(0.28—0.37)	6	0.17(0.16—0.17)	0.25(0.24—0.26)	3	0.29(0.27—0.30)	0.11(0.10—0.12)	2
0.46(0.40—0.50)	0.54(0.51—0.58)	5	0.19(0.17—0.24)	0.08(0.07—0.09)	3	0.46(0.44—0.48)	0.31(0.30—0.32)	2
			0.24(0.21—0.25)	0.22(0.21—0.22)	3	0.55(0.54—0.55)	0.75(0.73—0.77)	2
			0.25(0.23—0.28)	0.27(0.27—0.28)	3	0.63(0.62—0.64)	0.61(0.57—0.65)	2
			0.28(0.28—0.29)	0.45(0.42—0.47)	3			
			0.53(0.52—0.55)	0.59(0.55—0.63)	4			

out of five) their presence should be considered normal. Their occurrence is perhaps connected with a particular structure of a segment of a polypeptide chain (or chains ?) in hemoglobin which enters the composition of Fraction O-1, from which F-4 was obtained. This question requires further study.

Thus, this group of experiments leads to the conclusion that pepsin digestion of hemoglobin, independent of its duration, produces in the lowest molecular weight fraction an accumulation of products of hydrolysis which are peptides giving spots with definite R_f values in chromatograms. A number of these spots are constant.

Secondary digestion of higher molecular weight peptides (Fractions O-1 and O-2) formed in pepsin hydrolysis of hemoglobin gives in great part peptides which in two-dimensional chromatography give the same R_f values. This shows a sufficiently good reproducibility of the results of pepsin digestion of hemoglobin.

Amino Acid Composition of Some Peptides

Since the chromatograms of the pepsin hydrolyzate show a series of peptide spots which are sufficiently constant, we can assume that these peptides are characteristic of the sections of the polypeptide chains which compose the molecule of hemoglobin. Their analysis presents the possibility of approaching a solution of the structure of this protein.

Some of the spots were removed from the chromatogram. The eluate was hydrolyzed and the amino acids in it were determined chromatographically.

It was shown that it was difficult to obtain homogeneous material for hydrolysis from one-dimensional chromatograms. The strips of paper cut from the wide chromatograms at the level of each spot, as a rule, gave several spots after elution and repeated chromatography. This confirms the work of Dean and Truter [6]. Thus, from three experiments with strips cut at the level of the spot with R_f 0.52-0.55 (solvent n-butanol–CH_3COOH–H_2O) after repeated chromatography of the eluate, only one showed one spot. In two experiments repeated chromatography of the eluate from the spot with R_f = 0.62-0.65 gave several peptide spots.

After hydrolysis of the eluate from the strips of the wide chromatograms which contained only one spot with R_f value 0.52-0.55 (corresponding to the R_f of phenylalanine) we found, along with the spot of unchanged phenylalanine, seven spots of amino acids (leucine, phenylalanine, valine, alanine, glutamic acid, glycine, lysine). We showed that we

were dealing with a heptapeptide or with a mixture of phenylalanine with a peptide or peptides.

A test hydrolysis of the complex spot with R_f = 0.62-0.65 (corresponding to leucine) gave, as expected, a large number (nine) of amino acid spots.

TABLE 3

Average R_f Values for Peptides Obtained in the Digestion of Hemoglobin (I) for 2-3 Hours, Two Experiments, Fraction O-1 (II) Five Experiments, Fraction O-2 (III) Three Experiments

Two-dimensional chromatography; solvents the same as in Table 2; front at a distance of 15.5-20.7 cm; temperature 16-19°

F-3 (I)		No. of expts. in which spots were found	F-4 (II)		No. of expts. in which spots were found	F-5 (III)		No. of expts. in which spots were found
Solvent 1*	Solvent 2*		Solvent 1	Solvent 2		Solvent 1	Solvent 2	
0.05	0.08	2	0.05	0.19	3	0.04	0.07	3
0.06	0.15	2	0.06	0.36	4	0.14	0.23	3
0.17	0.13	2	0.09	0.25	3	0.17	0.13	3
0.17	0.30	2	0.15	0.13	4	0.20	0.27	3
0.21	0.30	2	0.14	0.22	5	0.26	0.15	3
0.38	0.19	2	0.17	0.30	5	0.26	0.40	2
0.36	0.45	2	0.30	0.38	3	0.34	0.36	3
0.51	0.55	2	0.31	0.30	3	0.53	0.56	2
			0.63	0.55	4	0.67	0.62	3
			0.71	0.75	3			
			0.53	0.54	2			

*Solvent 1 = n-Butanol – Acetic acid – H_2O; Solvent 2 = Alcohol – NH_3 – H_2O.

Note: R_f of single spots not shown.

To obtain a purer peptide we used two-dimensional chromatography. Material from three experiments was submitted to chromatographic separation: 1) Fraction F-3 from a typical one-hour experiment of pepsin digestion of hemoglobin, 2) Fraction F-3 from a three-hour digestion, and 3) Fraction F-5 from a one-hour digestion of Fraction O-2.

The results of the analysis of the hydrolyzate are given in Table 4.

First of all, it is apparent that there is sufficiently good agreement in the amino acid composition of the spots which have the same R_f values but come from different experiments.

Among the isolated peptides the minimum length ranges from a tripeptide to an octapeptide (assuming conditionally that there is one residue of each amino acid in the composition).

Most of the peptides which we have studied contain the amino acids leucine, valine, alanine, glutamic acid, glycine and lysine. In addition, we find peptides which contain tyrosine, phenylalanine, serine, aspartic acid and methionine.

DISCUSSION OF THE RESULTS

Until recently there was no information in the literature on the composition of the separate peptides which built up the hemoglobin molecule. Such a study is opportune because of the relatively low molecular weight of these proteins (66,000-67,000) and the evident comparatively simple structures of these molecules in several animals (cows, sheep, goats) where the polypeptide chains probably have valine and methionine as the N-terminal amino acids (in other animals such as horses and dogs, and also humans, only valine occurs) [7].

A recent short communication of Brown [8] describes the isolation of a series of cysteinyl peptides from the products of acid hydrolysis of human hemoglobin, and also peptides which contain a cysteic acid residue, obtained from the pepsin hydrolyzate of this protein. Only one of the groups of "pepsin" polypeptides had the size of a pentapeptide. The others had polypeptide chains of nine or more amino acid residues.

In the present work we studied the fractions with the smallest polypeptides. The largest of them contained eight amino acid residues (R_f = 0.15/0.18). Most of the peptides isolated by Brown probably occurred in Fractions

TABLE 4

Amino Acid Composition of Hydrolyzate Spots

Material	Rf values for peptide spots							
	0.52–0.54 / 0.53–0.56	0.33–0.36 / 0.45–0.49	0.25 / 0.27	0.14 / 0.27	0.15 / 0.18	0.16–0.17 / 0.12	0.12 / 0.07	0.04–0.05 / 0.16–0.18
F-3 (digestion 1 hour)	Leucine, valine, alanine, glutamic acid, glycine, lysine	Valine, alanine, glutamic acid, glycine, lysine	Leucine, methionine, tyrosine, alanine, glutamic acid, glycine, lysine	—	Leucine, valine, tyrosine, glutamic acid, glycine, serine, aspartic acid, lysine	—	Glutamic acid, glycine, lysine	Leucine, valine, tyrosine, glutamic acid, glycine, aspartic acid, lysine
F-3 (digestion 3 hours)	Valine, alanine, glutamic acid, glycine, lysine	Valine, alanine, glutamic acid, glycine, lysine	—	—	—	Phenylalanine, alanine, glutamic acid, glycine, lysine	—	Leucine, valine, tyrosine, glutamic acid, glycine, aspartic acid, lysine
F-5	Leucine, valine, alanine, glutamic acid, glycine, lysine	—	—	Leucine, alanine, glutamic acid, glycine	—	Phenylalanine, alanine, glutamic acid, glycine, lysine, serine	—	—

TABLE 5

Summary of Spots Found in All Series of Experiments

Series No.	F-3		F-4		F-5		Hydrolyzed spots	
	R_f	No. of expts. out of 6 in which spots are found	R_f	No. of expts. out of 5 in which spots are found	R_f	No. of expts. out of 3 in which spots are found	R_f	Amino acid composition
1	0.04/0.10	3	0.03/0.06	1	0.04/0.07	3	—	—
2	0.09/0.18	5	0.05/0.19	3	0.06/0.20	1	0.04/0.17	Leucine, valine, tyrosine, glutamic acid, glycine, aspartic acid, lysine
3	0.15/0.10	2	0.15/0.13	4	0. 7/0.13	3	0.17/0.13	Phenylalanine, alanine, glutamic acid, glycine, lysine (serine)
4	0.14/0.19	6	0.14/0.22	5	0.14/0.23	3	0.15/0.18	Leucine, valine, tyrosine, glutamic acid, glycine, serines, aspartic acid, lysine
5	0.12/0.31	3	0.16/0.30	5			0.14/0.27	Leucine, alanine, glutamic acid, glycine
6	0.22/0.14	6	0.21/0.18	2	0.26/0.15	3	—	
7	0.24/0.22	3	0.24/0.28	1	—	—	0.25/0.27	Leucine, methionine, tyrosine, alanine, glutamic acid, glycine, lysine
8	0.21/0.32	5	0.17/0.30	5	0.19/0.27	3	—	—
9	0.24/0.35	5	0.29/0.38	3	0.26/0.40	2	—	—
10	0.33/0.32	6	0.31/0.30	3.	0.34/0.36	3	—	—
11	0.41/0.54	5	0.47/0.48	1	—	—	—	—
12	0.53/0.59	4	0.52/0.54	2	0.54/0.56	2	0.53/0.55	Leucine, valine, alanine, glutamic acid, glycine, lysine
13	0.63/0.61	2	0.64/0.55	4	0.67/0.63	3	—	

O-1 and O-2 of our work which we have not studied in this paper.

Table 5 gives values of R_f for peptide spots which are met most often in pepsin digestion both of whole hemoglobin and of the intermediate products of its hydrolysis (Fraction O-1 and O-2)

Table 5 shows that the different peptides formed from whole hemoglobin and the products of its hydrolysis are often unlike. Thus, the peptide with R_f 0.41/0.54 found in most experiments on splitting whole hemoglobin is rarely formed when Fractions O-1 and O-2 are split. We therefore have the impression that those peptides which are split off at the beginning of digestion are later subjected to still deeper splitting and in every case they are not split from the fractions more stable to digestion which are obtained from the products of pepsin hydrolysis of hemoglobin (Fractions O-1 and O-2).

The idea of the hydrolyzing action of pepsin on some peptides which are not precipitated by 0.78 M CCl_3COOH can be considered probable enough. Some of the peptides in Fraction F-3 consist of seven or eight amino acids. Some of these (phenylalanine, tyrosine, alanine, glutamic acid, leucine), united by a peptide bond to a second component assure the splitting of this bond by pepsin [9-14]. Direct study of the liberation of amino acid nitrogen during long pepsin digestion of proteins (fibrin, gelatin, casein) has shown that it continues to increase even when the portion not precipitated by 20% trichloroacetic acid makes up 85.1-99.3% of total protein nitrogen after digestion [15]. This increase can be explained only by continued splitting off of low molecular weight peptides.

Thus, to consider the mixture of peptides which are not precipitated by 0.78 M CCl_3COOH as the "final" product of pepsin digestion can only be a first approximation.

It is especially necessary to remark that the qualitative determination of the amino acids in the peptides shows that the latter contain from three to eight amino acid residues. Since the quantitative content of the amino acids in the peptides is unknown, the size of the peptides (from tri- to octapeptides) calculated on this basis is a minimum. It is interesting that in the composition of Fraction F-3, which contains on the average tetrapeptides, there may be molecules with twice as many amino acid residues. This can be explained by: 1) the considerable solubility of the polypeptides in Fraction F-3 in spite of the large number (seven to eight) of amino acid residues which occur in its molecules; 2) the accumulation of excess lysine in F-3 which makes the calculation of the "average" size of the peptides on the basis of amino nitrogen content incorrect. The inhomogeneity of Fraction F-3 established in the previous communication [2] is clearly shown also in this work.

Finally, when the pepsin-digested Fraction O-1, which is made up on the average of dodecapeptides, is chromatographed, it shows several peptide spots with R_f values not found on the chromatograms from the other fractions; this fact indicates that there is some particular structure in the part of the hemoglobin from which this fraction is formed. It may be that the peptides which give these spots are formed by digestion of whole hemoglobin, but in this case their concentration in solution is slight so that they do not give a clear color with ninhydrin.

Desnuelle and co-workers [3] reported finding free phenylalanine in the pepsin hydrolyzate of horse globin. In our experiments on the digestion of hemoglobin, spots with the R_f of phenylalanine are often found. However, they are spots which correspond to peptides. It is known [16] that many peptides in chromatography have R_f values which correspond to the R_f values of various amino acids. Such effects have been seen in our work. Can this explain the contradictions with the work of the school of Desnuelle? Is the preliminary splitting of hemo from globin significant here, or the nature of the hemoglobin preparation (in our experiments we used commercial hemoglobin, apparently from cattle but not accurately known)? These questions require further study.

SUMMARY

After pepsin digestion of hemoglobin the low molecular weight fraction of the hydrolyzate gives by the method of two-dimensional chromatography more than 20 peptide spots. Eight spots regularly appear in parallel experiments.

Variations in length of digestion and digestion of intermediate products of pepsin hydrolysis of hemoglobin result in general in the appearance of the same peptide spots.

The eight spots have been submitted to qualitative analysis for amino acid composition. They contain from three to eight amino acids.

Spots which have R_f values resembling the R_f value for phenylalanine are due to peptides and not free amino acids.

Received October 6, 1957

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THE ROLE OF INTRACELLULAR PROTEASE OF CL. OEDEMATIENS IN THE FORMATION OF TOXIN

T. M. Manuil'skaia and V. A. Blagoveshchenskii

Division of Biochemistry, N. F. Gamaleia Institute of Epidemiology and Microbiology, USSR Academy of Medical Sciences, Moscow

Most of the known bacterial toxins are proteins and so proteases may be assumed to share in the biosynthesis of the toxins.

Dernby and Walbum [1] studied the proteolytic enzymes of diphtheria bacilli and expressed the opinion that participation of the proteases was possible in the formation of toxin. Smirnova [2] showed experimentally the relation between the activity of intracellular protease and toxin formation in Corynebacterium diphtheriae.

Up to now there has been no information in the literature on the connection of proteolytic enzymes with the formation of toxin in pathogenic microbes of the anaerobic group. A little studied member of this group is Cl. oedematiens, one of the producers of gas gangrene.

The purpose of the present work is to study the participation of intracellular proteases of Cl. oedematiens in toxin formation.

METHODS

Cl. oedematiens (toxigenic strain No. 794 and non-toxigenic strains No. 2 and 580) were cultured on a casein medium at 37° for 4 days. Samples were taken out each day to study the activity of Cl. oedematiens protease in the dynamics of toxin formation. The microbial mass which was separated from the culture medium was centrifuged and washed, and the enzyme activity was determined in it. For this purpose, 10 ml of phosphate buffer, pH 7.8, and 2 ml of 2% casein solution were added to 4 ml of a 10% bacterial suspension which had been ground with glass. The mixture was incubated under toluene for 24 hours at 37°. The activity of the enzyme was estimated by the increase in amino nitrogen which was not precipitated by 10% trichloroacetic acid. This was determined colorimetrically with Nessler reagent after digesting with H_2SO_4. The protease activity was expressed in mg % nitrogen per absolutely dry material of the microbe mass.

The content of toxin in the culture medium was determined by titration on mice and expressed in LD_m per ml.

EXPERIMENTAL PART

Figure 1 shows the data for the proteolytic activity of toxic strain No. 794 and nontoxic strain No. 580 of Cl. oedematiens. As is clear, the protease activity of the nontoxic strain is considerable above that of the toxic. Nontoxic strain No. 2 of Cl. oedematiens at the beginning of work with it also had high proteolytic activity, but with each transfer on a casein medium strain No. 2 became more toxigenic. The activity of the protease fell sharply with increasing toxicity of the strain. These data are presented in Fig. 2.

As Fig. 1 (solid line) shows, the beginning of toxin formation in Cl. oedomatiens strain No. 794 on the first day agrees with the maximum activity of the protease. Fall in proteolytic activity to the fourth day is accompanied by an increased titer of toxin in the medium.

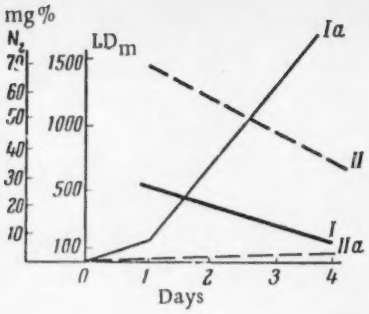

Fig. 1. Proteolytic activity in toxic and nontoxic strains of Cl. oedematiens. I) Protease activity in toxic strain; II) the same in nontoxic strains. I a) LD_m of toxin of toxic strain; II a) the same for nontoxic strains.

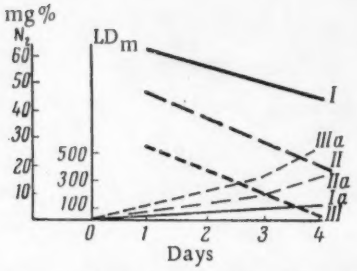

Fig. 2. Proteolytic activity of weakly toxic strain of Cl. oedematiens as it acquires toxic properties. Protease activity: I) at first transfer; II) at second transfer; III) at third transfer; Ia, IIa, IIIa) corresponding LD_m of the toxin.

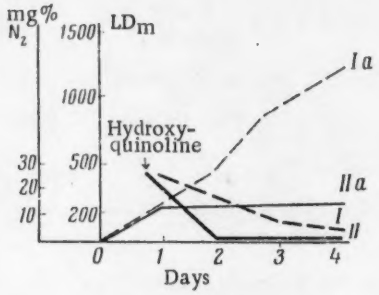

Fig. 3. Blocking of Cl. oedematiens protease by hydroxyquinoline in vivo. Protease activity: I) control; II) experiment with hydroxyquinoline; Ia, IIa) corresponding LD_m of toxin.

In order to show the relation between toxin formation and proteolytic activity of Cl. oedematiens, we carried out experiments with the addition of hydroxyquinoline, one of the inhibitors of proteolytic enzymes. Hydroxyquinoline in a concentration of 0.008 M was added to the culture medium a day after inoculation at the beginning of toxin formation.

As Fig. 3 shows, in these experiments the blocking of protease with hydroxyquinoline was accompanied by suppression of further toxin formation, that is, the toxin titer remained at the level of the first day, while in the control there was a sharp increase in the toxin titer of the medium. Thus the connection between protease and toxin formation in Cl oedematiens is evident.

DISCUSSION OF RESULTS

When we compare our results we see most clearly an inverse relation between toxin formation and protease activity in Cl. oedematiens. The high activity of protease on the first day corresponds to a low titer of toxin in the medium, and the decrease in proteolytic activity to the fourth day corresponds to a sharp rise in toxin titer.

The absolute value of protease activity in the nontoxic strain is higher than in the toxic. Increase in the toxigenic properties in the weakly toxic strain during the inoculation process is accompanied by fall in proteolytic activity.

When the Cl. oedematiens protease is blocked by hydroxyquinoline the formation of toxin stops entirely.

There are two possible assumptions which explain these facts. First, we can consider, in terms of the ideas of Oparin and Kursanov [3], that in microbe cells, just as in plant cells, protease has a simultaneous hydrolytic and synthetic function and takes a direct part in the synthesis of the toxin. Disturbance of equilibrium in these two mutually contradictory processes leads to the observed inverse relationship between protease activity and amount of toxin formed in the culture. On the other hand, we can assume that the Cl. oedematiens protease

in the cells liberates toxin from its bond with protein or nuclear systems. The possibility is also not excluded that this process may be complicated by a direct action of the protease on the toxin. In this connection, the study of the weakly toxigenic strain is especially interesting. The solution of these questions may be given by further experimental study of the relation of toxin formation to the activity of the intracellular protease under conditions of activation and inhibition of the enzyme.

SUMMARY

We have established a relation between protease activity and toxin formation in Cl. oedematiens. The maximum proteolytic activity corresponds to the beginning of toxin formation. A fall in enzyme activity to the end of the culture period is accompanied by a rise in toxin titer in the medium.

A study of the nontoxigenic strain of Cl. oedematiens shows that it has a higher proteolytic activity than the toxigenic strain.

When the Cl. oedematiens protease is blocked in experiments in vivo, the toxin titer does not increase from the moment of adding hydroxyquinoline, but remains at its original level.

Received October 30, 1957

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LABELLED METHIONINE IN THE INVESTIGATION OF SARCOPLASMIC PROTEIN

B. S. Kasavina and M. V. Umanskaia

Biochemistry Department, The Central Institute for Traumatology and Orthopedics, The USSR Ministry of Health, Moscow

Certain differences in protein composition between the myofibrils and sarcoplasm of the skeletal muscles of human, animal and bird embryos and adults were discussed in previous communications from this laboratory, dealing with the proteins of muscular tissues during the ontogenesis of man and other animal species [1-3]. A certain common pattern was demonstrated in the developing embryos of the various species, consisting in the accumulation in the muscle of the contractile protein, actomyosin, the decrease in myoalbumin content, and the development of the enzymatic systems of the sarcoplasm.

Other workers [4] have also demonstrated the occurrence of certain shifts during the ontogenesis of muscle tissue, when actual quantitative changes of the muscle protein themselves occurred.

In the present work, amino acid incorporation into proteins and into enzymes was studied with the aim of elucidating whether the differences in sarcoplasmic protein composition, observed during ontogenesis, was due to changes in protein synthesis rates. The method employed was a combination of electrophoresis and radioactive labelling, which allowed the investigation of the ratios of the various protein fractions of the sarcoplasm (aldolase, glyceraldehyde dehydrogenase, phosphorylase, myoalbumin), as well as their synthesis, inasmuch as the incorporation of labelled methionine reflected the rate of protein synthesis.

It will be shown below that the rates of sarcoplasmic protein synthesis varied in dependence of the age and species of the animals.

METHODS

The experimental animals employed were female rats or guinea pigs, 19-20 and 60-62 days pregnant, respectively (duration of pregnancy in rats – 21-22 days, in guinea pigs – 62-64 days). The animals received S^{35}-methionine subcutaneously, in amounts ranging from 50 to 100 thousand cpm per g body weight. Animals were killed 18-20 hours after the injection, the muscles of the extremities were removed from both the embryos and the parents; after removal of fat and connective tissues the muscles were finely divided with scissors and ground in the mortar*. The homogenate thus obtained was extracted with phosphate buffer of low ionic strength (0.1), pH 7.7. The extract, containing the soluble proteins and enzymes, was fractionated by paper electrophoresis into several fractions (aldolase + glyceraldehyde dehydrogenase, phosphorylase, myoalbumin), using a procedure described earlier [5, 6, 2]. The developed electrophoregrams were used for the determination of radioactivity (the various spots corresponding to the protein fractions), for the study of distribution of the various fractions and their rate of S^{35} incorporation. The electrophoregrams were cut into sections corresponding to the individual fractions, and radioactivity in each fraction determined using the apparatus B.

The aldolase and glyceraldehyde dehydrogenase fractions from adult muscle could be separated quite satisfactorily by paper electrophoresis, but only a poor separation was obtained under the same conditions with embryonal muscle fractions. The combined radioactivity of these two fractions was therefore assayed throughout.

*The age of embryos was determined from their weight and length, according to the table compiled by Needham [7].

It should be noticed that S^{35}-methionine incorporation into these fractions was found to be very slight. Direct label incorporation into muscle extract (total) was also studied.

Presented in Table 1 are the results of several experiments on S^{35}-methionine incorporation into the rat proteins (each experiment involved 15 animals, the individual results falling very close together).

TABLE 1

The Intensity of Labelled Methionine Incorporation Into the Proteins of the Rat

Age of embryo in days;	cpm per 0.1 ml of the extract of adult muscle;	cpm per 0.1 ml of the extract of embryonal muscle;	Methionine found in the electrophoregram fractions, cpm					
			adult animal			embryo		
			aldolase + glyceraldehyde dehydrogenase	phosphorylase	myoalbumin	aldolase + glyceraldehyde dehydrogenase	phosphorylase	myoalbumin
20	440	3306	11	8	2	30	23	35
21	1442	3857	31	23	18	173	201	98
21	600	2352	38	25	19	182	149	80
21	1005	6805	27	17	13	69	129	69

The amount of labelled methionine incorporated, as assayed per 0.1 ml of the dried extract, was found to be from 2.5 to 8 times higher in the embryo, as compared with the adult female (for instance, 2352 cpm, as compared with 600 cpm).

TABLE 2

The Intensity of Labelled Methionine Incorporation Into Guinea Pig Proteins

Age of embryo in days;	cpm per 0.1 ml of the extract of adult muscle;	cpm per 0.1 ml of the extract of embryonal muscle;	Methionine found in the electrophoregram fractions, cpm					
			adult animal			embryo		
			aldolase + glyceraldehyde dehydrogenase	phosphorylase	myoalbumin	aldolase + glyceraldehyde dehydrogenase	phosphorylase	myoalbumin
64	315	961	15	12	4	73	51	24
57	701	2289	4	4	3	29	36	41
64	645	4185	10	5	10	69	38	31

The same pattern was observed when the radioactivity distribution was determined in the various fractions on the electrophoregrams, namely, aldolase + glyceraldehyde dehydrogenase (m + 1 + n), phosphorylase (k) and myoalbumin (h)*. The labelled methionine incorporation into the various embryonal muscle fractions was much higher than its incorporation into the corresponding fractions from the adult females. Thus, in the adult rat, the incorporation factors for the three fractions, aldolase + glyceraldehyde dehydrogenase, phosphorylase and albumin, were 31, 23 and 18 cpm, respectively; the corresponding values for the embryonic tissues were 173, 201 and 98 cpm, respectively. The figures may be interpreted to indicate that, in the embryonal muscle sarcoplasm, methionine was incorporated into the protein at a rate 5-9 times higher than in the adult muscle.

Similar results were obtained with pregnant guinea pigs after administration of labelled methionine (Table 2). There were, however, certain noticeable differences involving species peculiarities.

Although in the guinea pig the proteins of the embryonal muscle were also found to incorporate labelled methionine at higher rates than those of the adult animal, the difference here was not as pronounced as in the rat. For instance, total methionine incorporation into the muscle extract was found to be 701 cpm in the adult

* The nomenclature adopted for the fractions was according to Toschi and Mariani [5-6].

animal and 2289 cpm in the embryo; similarly, a higher incorporation rate in the embryo, compared with the adult, was observed in the individual fractions, when their radioactivities were assayed on the electrophoregrams.

A comparison of results obtained with the rats and guinea pigs, respectively, showed certain noticeable differences in the incorporation of labelled methionine into the individual protein and enzyme fractions of the two species, as assayed by electrophoresis (Fig. 1). It was found that the various sarcoplasmic protein fractions of the rat incorporated the label at a much higher rate than the corresponding fractions of the guinea pig. This indicates that the synthesis of sarcoplasmic proteins in the embryos of the two species, during the last stage of their development, proceeded with different degrees of intensity.

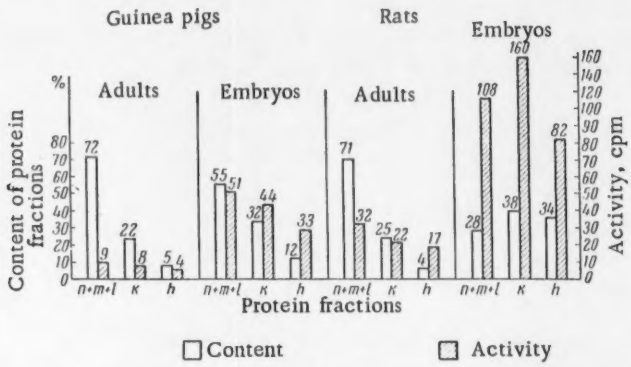

Fig. 1. Sulfur incorporation into protein fractions after S^{35}-methionine administration to pregnant rats and guinea pigs.

DISCUSSION OF RESULTS

The occurrence of qualitative and quantitative changes of proteins and of certain enzymatic systems in the muscles of developing animal embryos has already been demonstrated in our previous communications. These changes involved both the contractile muscle fibrils (actomyosin complex) and the soluble sarcoplasmic proteins. It was next considered of importance to study the incorporation of S^{35}-labelled methionine into the soluble proteins of the sarcoplasm. This incorporation, as well as the distribution of the various fractions [aldolase + glyceraldehyde dehydrogenase (m + l + n), phosphorylase (k) and myoalbumin (h)] was investigated by the combination of the radioactive labelling and paper electrophoresis methods; the intensity of S^{35}-methionine incorporation into any one protein fraction was then used as an indication of the intensity of its synthesis.

Radioactivity determinations on the protein fractions from both the embryo and the adult female showed that the label was incorporated into all the fractions investigated.

The results obtained also indicated that the processes of protein synthesis proceeded at considerably higher rates in the embryonal sarcoplasm, as compared with that of the adult.

Radioactivity measurements of the developed electrophoregrams obtained from the two animal species investigated showed that label incorporation into the various protein fractions of the embryo proceeded at higher rates than it did in the adult muscle. Thus, in the case of the fraction aldolase + glyceraldehyde dehydrogenase, the amount of activity on the corresponding spot on the paper was, for the embryo, about six times that of the adult female; in the case of phosphorylase, the factor was nine times, and with myoalbumin – five times; it is also of interest to note the differences in total label incorporation, into muscle and generally into the various fractions, between the different species of embryos investigated (guinea pigs and rats).

The observed difference in the rates of labelled methionine incorporation into the muscles of rats and guinea pigs indicates the different rates of protein synthesis in the two species (the process was found to be more intensive in the rat than in the guinea pig). These differences were found to be even more pronounced on comparison of the embryos of these two species. The embryos of blind-born animals, incapable of independent existence, showed a higher degree of radiomethionine incorporation into the sarcoplasmic proteins, and

this has been taken as an indication of intensive synthetic processes in the latest stages of the embryonal development in the rat. A different picture was observed in the newly born guinea pig, which is immediately capable of independent existence. When compared at the same developmental stage, guinea pig embryos showed a considerable lower intensity of radiomethibnine incorporation then did the rat embryos.

It may be concluded from the results quoted above that the biosynthesis of the proteins under investigation was more pronounced in the rat embryo than in the guinea pig embryo. The data are in good agreement with our previous results [6] concerning the formation of the actomyosin system during the ontogenesis of the rat and the guinea pig.

A comparison of the distribution of the various proteins with the incorporation of the S^{35}-methionine into the individual fractions showed that, under nearly identical conditions, the label was incorporated consistently more intensively into the rat tissues than in the guinea pig. This may probably be taken as an index of the different functional activity of the adult rat and guinea pig muscular tissues.

It follows from the results presented that, with regard to the intensity of methionine incorporation, the various fractions varied individually. This could best be demonstrated by the fact that the ratio of total activities of the fractions did not coincide with the corresponding quantitative ratios of the same fractions. There was a tendency for a higher specific radioactivity in the phosphorylase and the myoalbumin fractions. This observation provided yet another indication about the well-known biological reactivity of these proteins in metabolism. A characteristic feature of the embryonal myoalbumin was the fact that both its total content in the muscle and its degree of methionine incorporation were higher than in the adult animal.

The elevated activity of myoalbumin observed in the present work may, perhaps, be taken as a confirmation of the hypothesis that this protein participates in the biosynthesis of the actomyosin complex of the muscle fiber.

SUMMARY

The investigation of the synthesis rates of certain protein fractions and enzymes of the muscular sarcoplasm (aldolase + glyceraldehyde dehydrogenase, phosphorylase and myoalbumin) was made possible by the combined use of electrophoresis and radioactive labelling.

The various fractions (isolated on electrophoregrams) were shown to differ in their intensity of S^{35}-methionine incorporation; this was taken as an indication of different rates of synthesis of the various proteins.

It was shown that individual fractions of the muscle extracts of embryos incorporated methionine more intensively than did corresponding fractions from adult muscle.

It was shown that in the muscles of rat embryos (just prior to birth) protein synthesis proceeded at a higher rate than in muscles of guinea pig embryos, the finding being in agreement with the peculiarities of the ontogenesis of these species.

Received November 12, 1957

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THE CONTENT OF GLUTATHIONE SULFUR AND ASCORBIC ACID IN POTATOES AFFECTED BY WART

D. V. Lipsits

Laboratory of Physiology and Biochemistry, All-Union Research Station for Potato Wart, Chernovtsy

Wart growth in the tissues of various organs of potato plants occurs as the result of penetration and growth of the soil fungus Synchytrium endobioticum (Schilb.) Perc.; it is one of the varieties of plant tumors.

This potato disease is found in several European countries, in the USA, and in western USSR, and is the cause of injury to potato production. The introduction into agriculture of wart-resistant varieties of potatoes is an important procedure in the fight against potato wart. In this connection there is great practical and scientific interest in the study of the biochemical mechanism of the resistance of potatoes to wart. We already know some facts [1-6] established under the doubtful "oxidative" and "polyphenol" hypotheses of potato resistance, which confirm the need for study of substances closely connected with growth processes — nucleic acids, sulfhydryl compounds, growth substances, and polyphenols.

In the present work we give data obtained by studying the metabolism of sulfur-containing compounds in potatoes affected by wart, and also the results of ascorbic acid determinations, since this acid is of great significance in the physiology of plant tissues and its metabolism is closely connected with glutathione metabolism [7-12]. The study of ascorbic acid in this case is of even greater interest since it continues the attempts to relate the resistance of the plants to the disease with the concentration of ascorbic acid [13] or the products of its oxidation [14].

MATERIALS AND METHODS

As experimental material we used five potato plants susceptible to wart ("Vale," "Vol'tman," "Lorkh," "Seianets 36," and "Ella") and three which were wart resistant ("Karnea," "Grentsmar," "Oktiabrenok"), grown under field conditions in soils infected with wart-producers and in uninfected soils, and also potato sprouts infected with wart by contact with pieces of fresh wart tissue. In one series of experiments we introduced into the leaves (second layer) solutions of methionine with labeled sulfur, and sodium sulfate (with specific activity 17 and 25 μC). In a series of experiments solutions of radioactive sodium sulfate with a specific activity of 120 and 250 μC were introduced into the soil of the container in which the plants were grown. The harvested plants were separated into organs, carefully washed with running water, cut in pieces, fixed in a drying oven for 15 min at 110°, dried, and ground. The resulting air-dried powder was used for analysis.

To determine the activity we used a counting tube in a type B apparatus. The sulfur content was determined by oxidizing it to sulfate by the method of Pochinok [15]. Partial fractionation of the sulfur-containing compounds with isolation of protein sulfur and compounds of sulfur soluble in trichloroacetic acid (TCA) was carried out according to Mothes [16]. For this purpose a weight of 1 g of material was put in a beaker and 30 ml of distilled water was added. Extraction was carried out at 80° for 15-20 min. When the solution had cooled we added several drops of 5% hydrochloric acid and 8 ml of 10% TCA. After 1 hour the mass was centrifuged, the residue was washed three times with water and transferred to a porcelain crucible for burning. The filtrate was diluted to 50 ml and divided into two parts. One part was treated with a solution of barium chloride to precipitate the sulfate sulfur, the other was ignited after evaporation ("soluble sulfur"). We determined the activity

of the precipitates of barium sulfate which included all the sulfur from these fractions. The rate of uptake of sulfur in the plant after adding the radioactive sulfur to the soil was established by periodic sampling of the leaves with a test drill whose diameter agreed with the stage of the target and the mica window of the counter.

To determine glutathione, besides the method of Pett [8], we also used the more accurate iodometric method of Binet and Weller [17] according to which glutathione is precipitated from the exactly neutralized TCA solution by cadmium lactate before titration.

The histochemical determination of the SH group was carried out according to Chevremont and Frederic [18]. Pieces of tissue were fixed for 2-3 hours in 5% formalin which contained 0.9% NaCl; after washing with distilled water, sections were cut on a freezing microtome with a thickness of 15-30 μ; they were washed with water and put into three containers with cooled solutions (prepared immediately beforehand) composed of one volume of 0.1% solution of potassium ferricyanide and three volumes of 1% solution of ferric sulfate. Treatment of the sections lasted 20 min; after treatment they were washed with water and mounted in balsam. Similar treatment was given control sections kept 1 hour in a saturated aqueous solution of mercuric chloride [19]. The relative content of SH groups could be estimated by comparing the intensity of the color in the different sections. The difficulty with this method is that the intensity of color in the sections increases with length of treatment. Hence it is necessary always to maintain the same conditions of treatment.

By the advice of M. M. Epshtein (Chair of Biochemistry, Kiev Medical Institute) we used a method which permitted a quantitative estimation of the SH groups in the tissue. Ten thin sections treated by the method of Chevremont and Frederic were washed with water and transferred to 2 ml of 0.1 N solution of NaOH; when they were stirred they were completely decolored. The sample was filtered through a paper filter; the filtrate was neutralized with 2 ml of 0.1 N HCl, 5 drops of a 1% solution of ferric chloride were added, and the solution was diluted with water to 7 ml. The colored solution was put in a cuvette (5 mm) of a FEK-M photoelectric colorimeter and the determination was carried out with a red filter.

Ascorbic acid (AA) was at first determined in a metaphosphoric acid extract by titration with Tillmans red. Later we feared that in such an extract the hydrogen ion concentration would be insufficient to prevent oxidation of AA and so for the preparation of the extract we used 2% HCl and also added to the cut-up tissue 30 mg of solid oxalic acid per g of tissue [20]. AA and dehydroascorbic acid (DAA) were thus determined.*

We took a 1 g sample to which we added 5 ml of 2% HCl and cut it up and carefully ground it, allowed it to stand for 5 min, poured the whole mass into a tube, and centrifuged it. The centrifugate was collected in a micropipet and titrated with 0.1 ml of dye until the rose color vanished. To determine DAA 2 ml of the centrifugate was placed in a tube and carefully treated with soda until evolution of bubbles stopped. At the same time a stream of hydrogen sulfide was passed through the solution (for 15-20 min). After this several drops of fuming hydrochloric acid were added, carbon dioxide was passed in for 10-15 min and the sum of AA and DAA was determined in the solution. For determination of DAA we also used the method of Schaffert and Kingsley [21]. Two g of material was ground with 0.5% solution of oxalic acid, transferred to a calibrated flask and diluted to 100 ml and then filtered. To 4 ml of the filtrate in a test tube we added one drop of an alcoholic solution of thiourea and 1 ml of a 2% solution of 2,4-dinitrophenylhydrazine in 9 N H_2SO_4. The tube was placed on a boiling water bath for 10 min, then placed in cracked ice and treated dropwise with 5 ml of 85% H_2SO_4. The tube stood for 10 min and then was submitted to photometric measurement. The control test was treated in the same way as the experimental, but we did not add the solution of dinitrophenylhydrazine to it.

We also ran a histochemical determination of ascorbic acid according to Barnet and Burn [19]. Sections of fresh tissues from the freezing microtome were treated with acid silver nitrate solution (5 ml of glacial acetic acid added to 100 ml of 5% aqueous solution of silver nitrate) for 5-10 min. Then the sections were treated in the dark with 5% ammonia for 5-10 min, washed in distilled water, and placed in glycerol.

RESULTS

On the basis of experiments carried out with a large number of plants (148) we obtained fully trustworthy data on the particular distribution of radioactive sulfur in potato plants infected with wart.

* The method was recommended to us by Prof. S. F. Shamrai (Chair of Biochemistry, Kiev Medical Institute).

In Tables 1, 2, and 3 we give some typical results of the experiments.

TABLE 1

Distribution of Radioactivity in the Organs of Potato Plants*

Activity in counts/min on 50 mg of powder

Plant Organ	Radioactive preparation		Plant Organ	Radioactive preparation	
	Sulfate	Methionine		Sulfate	Methionine
Healthy young tuber	116	137	Healthy stolon	459	430
Affected young tuber	441	208	Affected stolon	760	525
Affected part of young tuber	680	348	Root neck	320	210
Parent tuber			Wart on root neck		
outer part	62	30	outer part	484	634
core	68	64	core	230	311
Wart on the tuber			Root	516	400
outer part	714	558	Leaf	359	420
core	448	205			

*Solutions of radioactive preparations with specific activity of 25 μC were placed on the leaves in the morning for 5 days (repeated four times.) The plant was gathered 10 days after beginning the experiment.

TABLE 2

Activity of Plant Samples 2 Days after Four Repetitions of Placing 15 Drops of a Solution of Radioactive Sodium Sulfate on the Leaves of Potato Plants Affected by Wart

Weight of sample 50 mg; specific activity of sodium sulfate 17 μC

Organ	Plant No.								
	1	2	3	4	5	6	7	8	9
Young tuber	34	42	33	33	48	50			
Wart on tuber									
Outer part	115	399	180	254	242	586	193	103	233
Core	48	95	57	162	81	235	72	61	112
Root	640	104	320	49	180	84.	111	448	950

As to the intensity of accumulation of radioactive preparations in the plants we could not find any difference, either between healthy and affected plants or a difference related to the introduction of the preparation. To a considerable extent the intensity depended on the method of introducing the preparation. When it was placed on the leaf the preparation reached the other plant organs and leaf stems much more slowly than when it was taken up by the root. On introduction of solutions of any of the radioactive preparations through the roots, measurable amounts of S^{35} appeared even in 2-2.5 hours in the upper leaves of young plants at a height of 50-60 cm, which indicates the rapid movement of sulfur compounds in plants.

An appreciable increase in content of radioactive sulfur was shown in affected organs of potato plants, and, especially, there was an increase in the wart tissue compared to normal tissue. The comparative radioactivity of the peripheral and core portions of the warts clearly showed the relation of the sulfur-containing compounds to the growth process. The peripheral part of the warts, composed of rapidly growing meristem tissue, attracted and accumulated sulfur compounds especially energetically.

Determination of the total sulfur content showed that there was considerable accumulation of these in the affected organs of potato plants, compared to the corresponding healthy organs (Table 4). This accumulation occurred, as the results of fractionation of the sulfur compounds showed, chiefly because of protein sulfur and

soluble organic sulfur compounds. Data which confirmed this were also obtained by measuring the activity of the barium sulfate precipitate which contained, as we already said, all the sulfur of these fractions.

TABLE 3

Activity of Plant Samples (50 mg) 2 Days after Adding 120 μC of Radioactive Sodium Sulfate to the Soil

Organ	Plant No.						
	1	2	3	4	5	6	7
Young tuber	64	94		193	216		167
Wart on tuber							
Outer part	1400	360	95	270	429	1162	3757
Core	283	285	63	261	72	166	825
			44	19	20	20	26
Parent tuber	32	21					
Stem	311	82	187	269	50	68	145
Root neck	238	71	187	211	100	86	223
Root	820	173	240	830	192	100	639
Leaf	325	133	171	270	94	70	123

TABLE 4

Distribution of Sulfur Fractions in the Organs of Potato Plants

Organ	Sulfur fraction in %			
	Total	Protein	Soluble organic	Sulfate
Healthy young tuber	0.198	0.066	0.073	0.059
Infected young tuber	0.228	0.09	0.095	0.045
Stem	0.492	0.08	0.31	0.102
Leaf	0.623	0.281	0.276	0.066
Healthy root neck	0.336	0.07	0.081	0.185
Infected root neck	0.446	0.092	0.252	0.102
Root	0.682	0.196	0.368	0.119

In the experiments using radioactive methionine the activity of the protein fraction, determined by the rate of its uptake, can to some degree serve as an indication of the rate of renewal and synthesis of proteins in the tissue. The data given in Table 5 obviously indicate an increased rate of these processes in the tissues of the plant warts and especially in their peripheral parts.

The considerable rise in glutathione found in the infected organs of potato plants (Table 6) fully agrees with the existing idea of the role of this tripeptide in the synthesis of proteins and is one more indication of its active share in the growth process.

The use of histochemical methods for studying glutathione and SH groups allowed us to establish that in healthy young tubers and also in infected tubers of resistant and susceptible types some amount of sulfhydryl compounds occur in the parenchymal layer directly adjoining the epidermis, and also in the epidermal cells and in the tissues which surround the vascular elements; considerably more SH groups were noted in young sprouts of 7, 13, and 20-day growth. We especially observed the sharp strengthening of the blue color in the cells of meristem tissues; SH groups accumulated at the top of the meristems of shoots and the points of growth from which rootlets were formed. We clearly observed the high concentration of SH groups in the secondary meristems which occurred under the influence of penetration of wart-producers into susceptible tissues. (Fig. 1). The tissues of the sprouts which grew profusely as a result of infection by wart stimulants and also the surfaces of the meristem tissue tuber-warts were colored dark blue, almost black, because of the high content of SH groups. No shift in content or localization of SH groups in infected sprouts of the resistant type was noted.

TABLE 5

Distribution of Sulfur by Potato Plant Organs and by Fractions, and also Activity of Fractions (in counts/min) 10 Days after Introducing Radioactive Sodium Sulfate or Radioactive Methionine through the Leaves

Organ	Total sulfur		Protein sulfur		Soluble organic sulfur		Sulfate sulfur	
	%	activity	%	activity	%	activity	%	activity
Sodium sulfate								
Healthy young tuber	0.185	1053	0.048	548	0.065	412	0.072	122
Affected young tuber	0.210	2240	0.044	1507	0.06	754	0.106	none
Parent tuber								
Core	0.116	398	0.05	228	0.031	120	0.035	94
Outer part	0.137	512	0.062	192	0.026	181	0.049	100
Upper layer of leaf	0.498	3102	0.248	2017	0.14	1024	0.110	236
Middle layer of leaf	0.436	3579	0.246	1976	0.112	1682	0.078	none
Root	0.236	2720	0.079	764	0.106	1216	0.051	645
Affected root neck	0.175	1728	0.036	320	0.097	659	0.042	680
Wart on root neck								
Outer part	0.260	2112	0.126	1519	0.086	956	0.048	none
Core	0.238	1614	0.144	1012	0.087	542	0.007	none
Healthy stolon	0.126	2457	0.092	901	0.029	920	0.005	461
Affected stolon	0.203	2602	0.089	2328	0.116	960	none	none
Wart on stolon, outer part	0.344	3665	0.10	2371	0.088	1048	0.156	574
Methionine								
Healthy young tuber	0.186	1784	0.064	926	0.064	632	0.058	24
Affected part of tuber	0.213	2792	0.09	1800	0.085	736	0.038	none
Tuber portion not affected	0.104	586	0.008	210	0.062	226	0.034	none
Parent tuber								
Core	0.184	558	0.045	128	0.059	57	0.08	324
Outer part	0.144	228	0.036	40	0.047	74	0.061	110
Upper layer of leaf	0.548	3885	0.333	2944	0.171	704	0.044	180
Middle layer of leaf	0.502	5184	0.255	3072	0.215	1280	0.032	233
Root neck	0.224	1920	0.045	320	0.09	768	0.089	88
Root	0.256	1900	0.112	536	0.106	736	0.038	148
Wart on root neck as a whole	0.288	4160	0.109	2560	0.120	1312	0 058	52
Stolons	0.212	3136	0.054	960	0.09	1179	0.068	208
Wart on stolons	0.320	3641	0.101	2368	0.133	964	0.086	174

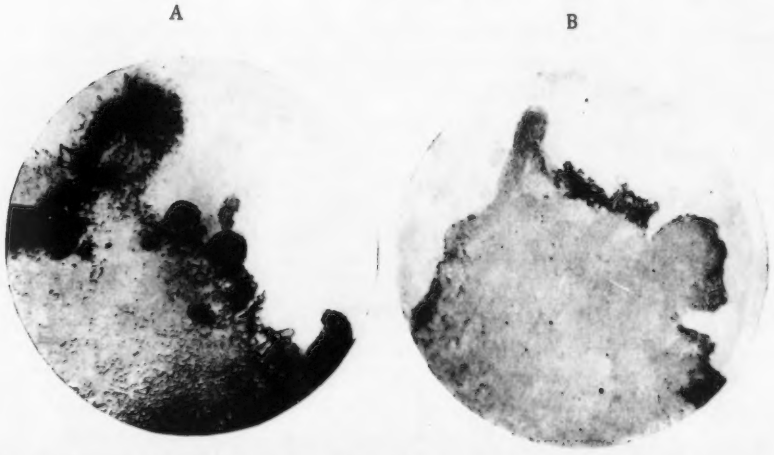

Fig. 1. Section of affected sprout, dyed according to Chevremont and Frederic to disclose SH groups (A), and control section treated before dyeing with mercuric chloride according to Chevremont and Frederic (B).

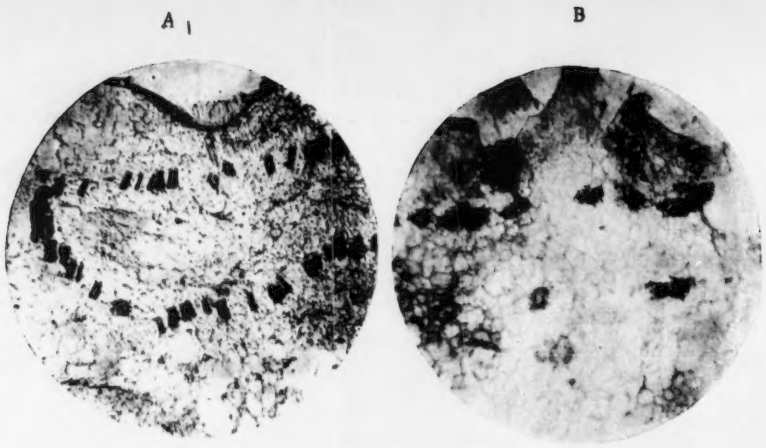

Fig. 2. Section of healthy shoot (A) and section of wart (B) treated by the method of Barnet and Burn to disclose ascorbic acid. Dark spots in Fig. 2, B are fungal spore cases.

TABLE 6

Glutathione Content in the Eye Tissue of Potato Tubers

In mg % of fresh weight

Type	Date of analysis	Tuber			Wart
		Uninfected	Infected	Attacked	
Susceptible					
"Vale"	15.VII	17.4	—	19.3	30.44
	10.VIII	14.0	18.0		25.66
"Vol'tman"	20.VII	13.26	18.9	23.6	25.2
Resistant					
"Grentsmark"	13.VII	16.75	15.96	—	—
	8.VIII	14.0	12.28	—	—
"Karnea"	18.VII	19.16	19.0	—	—
	13.VIII	12.95	15.66	—	—

Quantitative estimation of the content of SH groups showed that in the infected part of the sprouts they were present in greater amount than even in actively growing normal tissue. Typical results of this determination are given in Table 7.

TABLE 7

Quantity of SH Groups in Sprouts

In scale units of the photoelectrocolorimeter

Portion of sprout	22 Day old "Vol'tman" sprouts		20 Day old "Vale" sprouts (attacked at base of sprout)
	Healthy	Attacked	
Top	11.2	16.5	9.9
Middle part	7.0	8.2	8.0
Base	3.0	2.4	14.2

Determination of AA showed first of all that in the extraction replacing metaphosphoric acid by hydrochloric acid and the addition of oxalic acid did not change the final result. Hence metaphosphoric acid, recommended by Prokoshev [22], can be used successfully for preparing extracts of plant tissues which have powerful oxidizing systems.

TABLE 8

Content of Ascorbic Acid and Dehydroascorbic Acid in Healthy and Attacked Sprouts and Tubers of Potatoes and also in the Warts

In mg % fresh weight

Material	AA	DAA	Material	AA	DAA
2 cm long sprouts of "Grentsmark"			Young tubers of "Vol'tman"		
upper part	16.2	3.8	healthy	19.3	0
base	9.0	1.5	infected	18.6	0.7
6 cm long "Grentsmark" sprouts			attacked	17.5	0.6
			Warts on young		
upper part	11.0	0.2	"Vol'tman" tubers	10.9	0.3
base	6.0	1.0	Young tubers of "Vale"	19.2	0
23 Day old "Vol'tman" sprouts			healthy	16.8	0
			infected	14.3	1.8
healthy	15.4	5.6	attacked	13.1	0.7
attacked	18.4	2.3	Warts on "Vol'tman" tubers, peripheral		
30 Day old "Ella" sprouts			part, core	9.0	0
			Warts on "Ella" tubers		
healthy	11.9	4.8	peripheral part	20.0	0.7
infected	13.0	3.7	core	10.1	0.4
attacked	18.5	5.2			

As Table 8 shows from the typical results of analyzing the plant samples, an increased content of AA is found in almost all cases where more active growth processes occur. Contrary results would appear to occur from the data on AA content in healthy and attacked young tubers and in the warts on these tubers. However, this contradiction is only apparent and disappears if we consider the change in content of dry substances in the tubers as a result of attack by the warts. In all cases contact of the susceptible type of potato tubers with the wart-producer leads to a smaller percent content of dry substance. Thus, for example, in healthy "Vale" tubers there is 21.5% dry substance; in infected tubers, 28.8%; in tubers attacked by warts, 18.9%; and finally, in the warts, 12.4%. When the data of Table 8 are calculated on dry weight the AA content in the warts, especially their peripheral parts, is raised considerably in the healthy young tubers (for example, in "Vale" tubers 89.28 and 105.58%).

The histochemical findings show that in healthy sprouts AA is concentrated at the bottom in the conducting tissues of the fibrovascular bundles (Fig. 2). In the shoots which have been attacked, the distribution of AA has a diffuse character which makes its quantitative determination and comparison difficult.

DISCUSSION OF RESULTS

As recent investigations have shown the synthesis of proteins is increased in wart tissues [23], respiration rises [5], oxidative enzymes are activated [1]; thus there is a general stimulation of metabolic processes. The considerable increase in sulfur content which we have shown for plant organs affected by warts and for the warts themselves is therefore quite natural, since this element is required for the synthesis of sulfur-containing amino acids, peptides, proteins, many enzymes and other biologically important substances [24].

The accumulation of glutathione in wart tissues must be considered as evidence for the participation of this substance in growth processes.

From the time of the classical work of Hammett [25] there have appeared many papers on the reciprocal relation between SH groups and growth [26-29], and the relation of SH groups and auxin during plant growth and development [30-32]. There are data on the increased content of SH groups in animal cancer tissues [33,34]. The same increase in SH content was observed by Binet and Magrou [35] in stems and leaves when the stems of some plants were inoculated with Bacterium tumefaciens and tumors developed. Important evidence on the decisive role of protein SH groups in growth was recently presented by Mazia [36].

The characteristic localization in the tissues of attacked sprouts of the SH groups, the change in their quantity during penetration of the wart producers and the appearance of deformation and growth indicate that in the development of these warts the structure of the protein substances plays a large part. Evidently, active growth processes presuppose a preliminary change in protein structure so that the quantity of SH groups, is changed; SH groups which activate the biological processes guaranteeing the appearance and development of the warts. These structural changes of protein caused by fungus penetration of the cell are possible, but it is not known why they occur only in the tissues of susceptible types of potato.

There is a large literature on the physiological function of ascorbic acid. In particular, it is generally known that this vitamin is found in increased amounts in areas of high metabolic activity in both plants and animals [37-40]. According to Prokoshev [41] the concentration of ascorbic acid is maximal in young, growing, or rapidly regenerating tissues and its content decreases as the tissue ages and loses its mitotic activity.

Our data indicate that potatoes attacked by warts agree with these general principles: rapidly growing wart tissue contains a large amount of ascorbic acid. The amount of dehydroascorbic acid is then very small, although it would seem that in the warts conditions would be favorable for functioning of the "polyphenol-oxidase-phenol" system which, according to Engel'gardt and Bukin [42], can easily oxidize ascorbic acid. Keeping it in the reduced form evidently assures the accumulation of glutathione in the wart.

SUMMARY

S^{35} in wart tissues accumulates more intensively than in normal tissues of the potato plant.

Organs attacked by wart and especially the warts differ from healthy organs by increased content of protein sulfur and glutathione, and also by increased radioactivity after introduction into the plant of sodium sulfate and methionine with labeled sulfur.

The rate of renewal and synthesis of proteins in the warts, determined by the rapidity of inclusion of methionine in the protein tissues, is high and surpasses the rate of these processes in all the other parts of the plant which we studied.

There is a close connection between sulfur-containing compounds and SH groups in the formation of wart growths in affected potatoes.

The affected tubers of the susceptible types of potatoes show a decrease in percent dry weight and in ascorbic acid.

In sprouts affected by warts and in the warts, especially their peripheral parts, where growth processes are most active, there is an increased ascorbic acid content (calculated on dry weight).

Received December 2, 1957

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A STUDY OF "ELECTROPHORETIC HOMOGENIZATION" OF PROTEINS

G. V. Troitskii and V. I. Okulov

Chair of Biological Chemistry, Crimean Medical Institute, Simferopol'

Proteins of blood serum show uniform electrophoretic changes under the influence of heating [1,2], ultraviolet radiation [3], treatment with some aldehydes and quinones [4,5], and with concentrated urea solutions [6]. In all these cases there is an increased content of the fraction which moves with the rate of α- or β-globulins; when the treatment is intense enough, the five or six protein fractions of serum are converted to give one electrophoretically homogeneous peak. This effect can be called "electrophoretic homogenization," by which is implied homogeneity from the point of view of electrophoretic analysis.

The study of "electrophoretic homogenization" is especially interesting in connection with the following.

1. For theoretical assumptions in the field of structure and denaturation of proteins.

2. For the reaction: albumin → α-(or β-)globulin ← γ-globulin, which is evidently related to the question of the origin of natural and pathological α- and β-globulins [7]. Since analogous changes in blood proteins are found in pathology [8], in perfusion through active organs of animals [7], and, it must be assumed, in connection with the transport function of proteins [9].

3. "Electrophoretic homogenization" is a characteristic change which blood proteins undergo in the preparation of blood substitutes from heterogeneous serum [10] and when plasma and serum are kept [11].

This investigation was undertaken to explain the mechanism of this protein transformation. It was interesting to explain the question of whether "electrophoretic homogenization" showed any secondary physicochemical effects which accompany denaturation, or whether it was the only existing evidence of the latter.

We made an electrophoretic study of denaturation effects in albumins and globulins from mixtures of types of proteins which differed in origin. Besides blood serum we studied extracts from different animal organs and one group of extracts from plant materials. We started from the assumption of a close relationship in the biosynthesis of albumins and globulins in any biological specimen and assumed that any mixture of albumins and globulins (of the same origin) would show after denaturation characteristic general modifications of form.

In addition, to explain the role of adsorption and aggregation in the mechanism of "electrophoretic homogenization" after treatment by heat and urea, we investigated the separate fractions of blood serum proteins. We also studied the electrophoretic properties of artificial mixtures.

EXPERIMENTAL

We studied the following protein preparations.

1. Blood serum of cows, swine, dogs and humans. We isolated by the method described earlier [12] with ammonium sulfate from beef serum: a preparation of albumin 90-93% electrophoretically pure; "globulin," a mixture of all the globulins in about equal proportions; electrophoretically pure γ-globulin.

2. Liver protein, obtained by fine grinding of pig liver tissue with M KCl [13] and concentration by precipitation with ammonium sulfate.

3. Protein from striated muscle of rabbits, obtained by the same method.

4. Protein from beef eyes obtained by concentrating the protein of the aqueous humor and the vitreous body. The protein from the initial solutions was precipitated by ammonium sulfate, the precipitate was dissolved in the minimal amount of distilled water and given a long dialysis in the cold, at first against a large amount of distilled water, and then against 1% NaCl solution.

5. The total protein of hen's eggs brought to the desired concentration with 1% NaCl solution.

6. Pea protein, obtained by finely grinding peas with a 10% NaCl solution and concentrating the extract as with the protein from beef eyes.

To reach about the same ionic strength as for serum, all the preparations were dialyzed against 1% NaCl solution.

2% solutions of protein were heated on a water bath with a thermostat in the limits $\pm 0.05°$ at various temperatures (50-65°) for 2 hours or were treated with various concentrations of urea (30-70 g per 100 ml, that is, 5 - 11.57 moles of urea per liter of serum) for 2 hours. Heating was carried out at different pH values which were obtained by adding different buffer solutions to the experimental and control samples. Then the experimental samples were treated and the controls were kept in the refrigerator. Immediately after treatment the experimental and control samples were simultaneously dialyzed against a veronal buffer and then analyzed electrophoretically.

We used the free boundary method of electrophoresis with optical recording which was described before [14]. For electrophoresis we used a veronal buffer with pH 8.6 and ionic strength 0.1. The pH of the samples and the buffer systems were controlled by a glass electrode. The conductivity of the veronal buffer and the dialyzed protein solutions at 4° was $0.0029 \pm 0.0001 \Omega^{-1} \text{cm}^{-1}$.

Calculation of the ratio of protein fractions and the movement of the separate fractions in the electrophoretic curve was carried out by the usual methods with a mechanical coulometer.

RESULTS

Blood Serum Proteins

The electrophoretic change in blood serum proteins after various types of treatment has been described by a number of authors [1-6]. Similar data have been given in another paper from our laboratory [16]. In this paper we give data from further experiments which permit us to compare the results for a number of sera and for their separate protein fractions.

In Fig. 1 we give the changes in content of blood serum protein fractions after heating and after treatment with urea at pH 7.0; Fig. 1 shows that with increased temperature of heating or urea concentration the α-globulin content is sharply increased* because of the decrease in all the other fractions.

In Fig. 2 (in series 1) we give the electrophoretic curves obtained by analysis of whole serum heated at pH 7.0 and 11.0 (60°, 2 hours). These data show that at pH 7.0 full homogenization of the protein mixture is not attained, and we find only a sharp increase in the content of the α_2-fraction because of decrease in the content of all the other fractions. If the serum is heated at higher temperatures (65°) we find a strong clouding of the solution.

When heating takes place at pH 11.0, "homogenization" is complete and only one peak occurs on the curve. This can be explained as the addition of the denatured substance itself to the medium; also such a strongly alkaline medium hinders aggregation of the denatured protein molecules with later coagulation.

In Fig. 3 for comparison we give the changes in electrophoretic curves after heating at pH 7.0 and pH 11.0 for whole serum [1], albumin preparation [2], "globulins" [3], and γ-globulin [4]. Evidently, albumin and γ-globulin, which have been isolated, behave in the same way as in serum, that is, albumin gives a slower and globulin a faster fraction. As a result both proteins give fractions which have very similar mobilities (in the case of albumin, a fraction with the mobility of α_2-globulin; in the case of γ-globulin, a fraction with the mobility of β_1-globulin). We found that the albumin preparation had greater stability toward heating compared to the albumin fraction in whole serum.

* In calling this fraction α-globulin here and in the future, we mean an electrophoretic term.

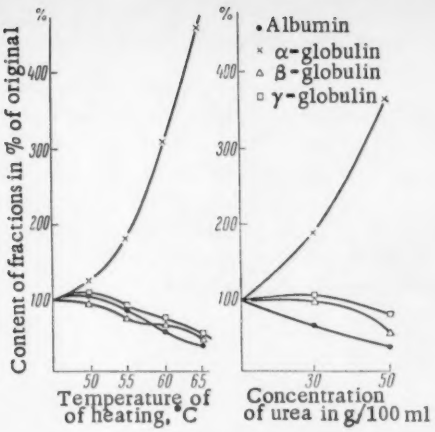


Fig. 1. Change in content of protein fractions of blood serum after heating and treatment with urea.

Fig. 4 gives the results of experiments in which albumin and globulin preparations were heated and then mixed.

Curves 1 and 2 are for the starting preparations, curves 3-8 are for the same preparations heated at pH 8.0 at 60° for 1 hour (medium degree of denaturing action, incomplete homogenization), and curves 9-12 for pH 11.0, 60° and 1 hour duration (complete homogenization). 2.5% solutions of these preparations were heated and cooled, then mixed in 1 : 1 ratio with different combinations: native albumin-native globulin, native albumin-denatured globulin, denatured albumin-native globulin, denatured albumin-denatured globulin.

The results of analysis of the curves show that electrophoretic changes of each preparation occur independently of the second component protein, whether it is denatured or native protein. Adsorption of native albumin by denatured globulin was not observed; the

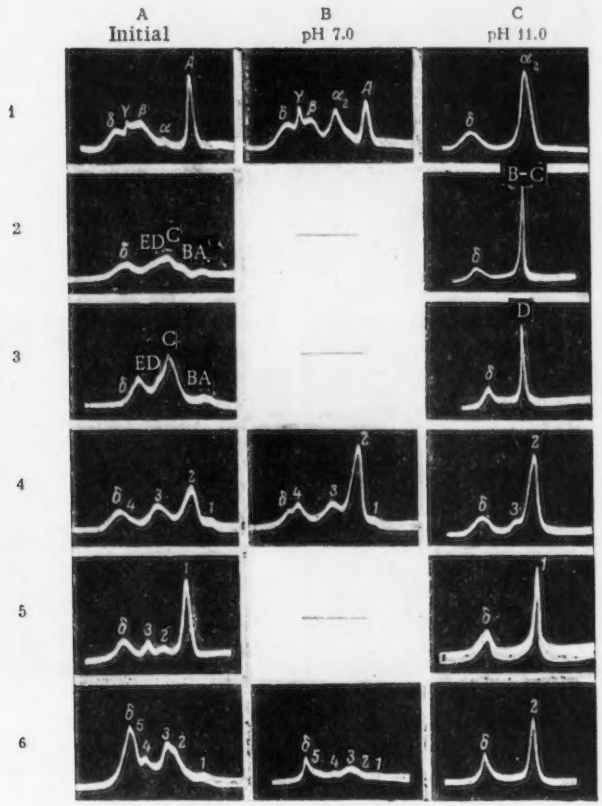
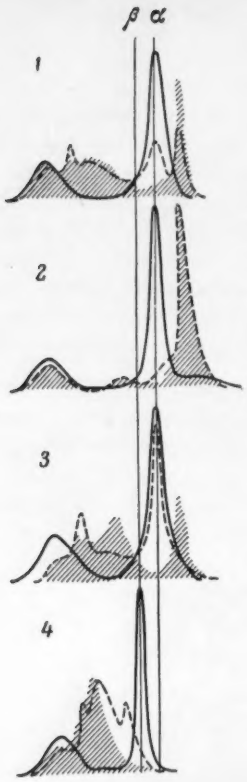


Fig. 2. Electrophoretic analysis of protein preparations heated at various pH values. 1) Beef blood serum protein, 2) swine liver protein, 3) rabbit muscle protein, 4) beef eye protein 5) hen's egg protein, 6) pea protein. A) Initial preparation, B) preparation heated at pH 7.0, C) preparation heated at pH 11.0, fully homogenized. The movement of the individual protein peaks corresponds approximately to the average movement of the proteins of each preparation. Designation of fractions, see text. δ — salt peak.



same could be said of all the other combinations.

Completely "homogenized" preparations (heated at pH 11.0) showed electrophoretically homogeneous peaks (curves 9 and 10) and when they were mixed, we also found only one homogeneous peak (curve 11). In the mixture of fully denatured globulin with native albumin (curve 12) there are two separate peaks in succession, and in this case adsorption of native albumin by denatured globulin was not found.

Fig. 3. Changes in proteins of beef blood serum and separate protein fractions after heating. 1) Blood serum; 2) albumin preparation; 3) "globulin" preparation; 4) γ-globulin preparation. A) Albumins; α, β_1, β_2, γ-globulins; δ) salt peak. The line α corresponds to the mobility of α_2-globulin; line β corresponds to the mobility of β_1-globulin; shaded area – starting curve, dotted lines, preparation heated at pH 8.0, solid lines, preparation heated at pH 11.0.

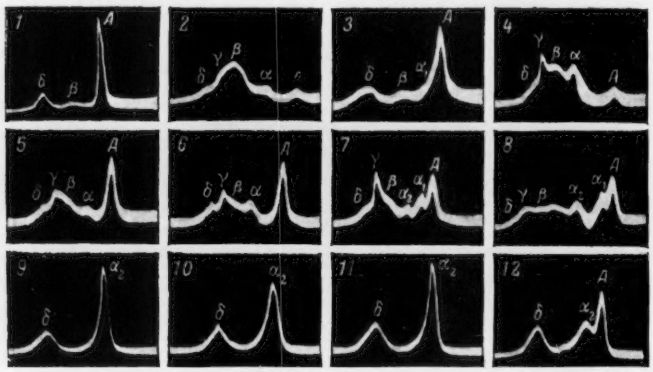


Fig. 4. Electrophoretic analysis of mixed protein fractions 1) Starting preparation of albumin; 2) starting preparation of globulins; 3) albumin preparation heated at pH 8.0 (60°, 1 hour); 4) globulin preparation heated at pH 8.0 (60°, 1 hour); 5) starting albumin + starting globulin (1 + 2); 6) starting albumin + heated globulin (1 + 4); 7) heated albumin + starting globulin (2 + 3); 8) heated albumin + heated globulin (2 + 4); 9) albumin preparation heated at pH 11.0 (60°, 1 hour); 10) globulin heated at pH 11.0 (60°, 1 hour); 11) heated albumin + heated globulin (9 + 10); 12) starting albumin + heated globulin (1 + 10). Letter designations the same as in Fig. 3.

Swine Liver Protein

The results of analysis of the starting preparations are given in Table 1. The five electrophoretic fractions are denoted by letters of the alphabet.

TABLE 1

Amounts and Mobilities of Protein Fractions from Swine Liver Extracts

Fraction	Mobility in $cm^2 \cdot sec^{-1} \cdot V^{-1} \cdot 10^{-5}$	Amount of fractions in %
A	7.4	3.0
B	5.8	16.7
C	4.4	41.0
D	3.4	22.6
E	2.3	16.7

TABLE 2

Amounts and Mobilities of Protein Fractions from Striated Rabbit Muscle

Fraction	Mobility in $cm^2 \cdot sec^{-1} \cdot V^{-1} \cdot 10^{-5}$	Amount of fractions in %
A	7.6	7.5
B	6.1	13.4
C	4.0	8.2
D	3.0	5.9
E	1.1	15.0

Because of the ease of precipitation of the extract proteins we did not succeed in obtaining a clear solution after heating in neutral or acid (pH 2.0) solution. In an alkaline medium (pH 11.0) heating 1 hour at 60° did not give a cloudy solution, and electrophoretic analysis of the heated solution showed the presence of one fraction which moved at a rate $5.1 \cdot 10^{-5}$ $cm^2 sec^{-1} \cdot V^{-1}$, that is, with a mobility about average compared to the mobility of all the other extract fractions.

The electrophoretic curves of the starting protein preparation and the preparation heated at pH 11.0 are given in Fig. 2, series 2.

Rabbit Muscle Protein

Analysis of the starting preparation showed five fractions, designated by the prime letters. In Table 2 we give the ratio and electrophoretic mobility of the separate fractions.

When the protein solution was heated in the neutral pH zone, it became cloudy. Electrophoretic analysis of the preparation heated at pH 11.0 and 60° for one hour showed a decrease in amount of fractions of two to three-fold and an increase in mobility of the remaining fractions. The fraction which earlier moved at a rate of $3.0 \cdot 10^{-5}$, after heating had a mobility of $3.5 \cdot 10^{-5}$ $cm^2 sec^{-1} \cdot V^{-1}$, and its relative content increased to 70-80%. Thus muscle protein after denaturation also has a tendency to the concentration of one middle fraction.

In Fig. 2, series 3, we give the electrophoretic curves of the starting and heated preparations.

Beef Eye Protein

In the starting preparation there were four fractions, of which the first was close in electrophoretic mobility to blood serum albumin and the other three to globulins.

In Table 3 we give the electrophoretic mobilities and ratios of these fractions, denoted by numbers.

We observed an increased mobility of all fractions after heating, and an increase in content of the fraction which moved at about the rate of fraction 2, namely, $5.0 \pm 0.5 \cdot 10^{-5}$ $cm^2 sec^{-1} \cdot V^{-1}$. Eye proteins were less stable than blood serum proteins: electrophoretic changes were noted even after heating to 45°.

Table 4 shows the changes in content of the separate fractions after treating them under different conditions.

In Fig. 2 , series 4, we give the electrophoretic curves obtained by analysis of starting and heated solutions.

Beef eye proteins undergo changes after denaturation which are analogous in principle to the changes of the protein preparations which have already been described: there is a tendency for all fractions to form one which approaches the "average" fraction.

TABLE 3

Amounts and Mobilities of Protein Fractions of Beef Eyes

Fraction	Mobility in $cm^2 \cdot sec^{-1} \cdot V^{-1} \cdot 10^{-5}$	Amount of fractions in %
1	6.0	11.8
2	5.0	41.0
3	2.5	33.2
4	1.3	14.0

TABLE 4

Change in Content of Beef Eye Proteins After Heating or Treatment with Urea
In % of the given fraction contained in the starting solution

Fraction	Heated				Treated with Urea (70 g/100 ml)
	pH 7.0			pH 11.0	
	45°	50°	55°	60°	
1	51.0	76.1	61.0	0	64.5
2	141.5	166.0	194.0	222.4	193.7
3	78.5	33.1	40.0	25.9	39 0
4	85.7	85.7	0	0	0

Hen's Egg Proteins

The electrophoretic mobility and percent content of the three fractions, which are clearly shown in the analysis of the starting protein are as follows:

Fraction	Mobility in $cm^2 \cdot sec^{-1} \cdot V^{-1} \cdot 10^{-5}$	Amount of fraction in %
1	6.8	73.0
2	4.0	13.0
3	4.5	14.0

The changes when hen's egg protein fractions are heated are analogous to those described for the above preparations: increase of one fraction at the expense of all the others and formation of one electrophoretically homogeneous component when the denaturing action is strong. Treatment with urea gives similar results. The characteristics of hen's egg proteins are the ease of precipitation when heated in a neutral or acid solution and the difficulty of attaining complete "homogenization:" a single peak is obtained only after two-hour heating in a strongly alkaline medium (pH 11.0-12.0). In addition, the mobility of the single peak is not that of the average mobility of all three fractions, but is close to the mobility of the first fraction and varies depending on the conditions of treatment within the limits $6.6\text{-}7.3 \cdot 10^{-5} cm^2 \cdot sec^{-1} \cdot V^{-1}$.

The electrophoretic curves for the starting and heated preparations are given in Fig. 2, series 5.

Pea Proteins

The composition of the protein extract depends to a considerable degree on the method of extraction, degree of grinding the pea meal, length of standing, etc. In the electrophoretic analysis of different preparations there may be from two to five protein fractions.

Table 5 shows the amounts and electrophoretic mobility of the different protein fractions in a preparation of pea protein which contains the largest number of fractions.

TABLE 5

Amount and Mobility of Pea Protein Fractions

Fraction	Mobility in $cm^2 sec^{-1} \cdot V^{-1} \cdot 10^{15}$	Amount of fractions in %
1	6.4	13.1
2	4.8	20.6
3	3.9	32.0
4	1.8	24.6
5	0.8	9.7

The most obvious property of the pea proteins is their tendency to coagulate with various treatments. In a neutral medium even at 45-50° part of the protein precipitates. Electrophoretic analysis of the filtrate shows about the same composition as in the starting solution, that is, the precipitate contains all the fractions equally. After heating in a strongly acid medium, pH 2.0, the solution remains clear, but during dialysis against a veronal buffer, that is, during passage through the isoelectric point, all of the protein precipitates. The precipitate also appears after treatment of the solution by various aldehydes, including formaldehyde which usually prevents aggregation and coagulation of proteins [17]. When the protein is heated in an alkaline medium (pH 9.0-10.0-11.0) the precipitate does not appear and electrophoretic analysis shows an increase in the content of one fraction with mobility of $5.0 \pm 0.5 \cdot 10^{-5} cm^2 sec^{-1} V^{-1}$. The transformation of all the fractions into one is shown more clearly in the more alkaline media; at pH 11.0 or higher we observed the presence of one electrophoretic peak. Urea behaved in a similar way, though less definitely.

The electrophoretic curves for the starting and heated preparations are given in Fig. 2, series 6.

Thus the electrophoretic changes of plant proteins after denaturation are analogous to those of animal proteins: in the cases studied the different fractions of the protein mixtures go over into one electrophoretically homogeneous form which usually has a mobility which is the average of the mobilities of all the starting fractions.

DISCUSSION OF RESULTS

We can conclude from the foregoing data that denaturation of very different types of proteins, plant and animal, leads to a shift toward electrophoretic uniformity, similar in nature with different methods of treatment and distinguished only by the degree to which it occurs. The ability to respond to denaturation by "electrophoretic homogenization" deserves serious consideration, since it may reflect important regularities in protein structure.

The nature of this phenomenon can be partly explained from the experiments which we have described and from the data in the literature.

A number of authors have explained the fact of the simplification of the electrophoretic composition of blood serum by the adsorption or aggregation of the different components of the protein mixture with resulting formation of a single complex from the electrophoretic point of view [1, 18, 19]. A somewhat modified version of this view holds that splitting of the protein molecule occurs and is followed by aggregation of the fragments [3, 19].

There is also the idea that splitting of lipoprotein complexes into the α- and β-globulins of blood serum lies at the base of "homogenization" [20, 21].

All these theories, in our opinion, have definite drawbacks.

Thus, the idea of aggregation of protein molecules as the basis of the "homogenization" process does not explain the reason for the effect, and particularly leaves unclear why the slower fractions increase their mobility (heating of homogeneous γ-globulin). It would seem that aggregation should always decrease electrophoretic mobility. In addition, such a denaturing agent as urea for the most part does not cause aggregation of protein molecules, or even causes splitting of some protein molecules [22, 23] and its action on proteins is analogous to the action of temperature. Finally, in a number of studies [24] it was found that high electrical charge of the protein molecule, and low concentration of protein and electrolyte can cause denaturation without aggregation; these conditions, as our experiments showed (heating at pH 11.0-12.0) not only do not hinder, but may even hasten the "homogenization" process. There is a suggestion [25] that in pH limits 4-9 serum albumin is broken up into finer particles and not aggregated. All these data lead to the opinion that aggregation merely accompanies "electrophoretic homogenization" under certain conditions, but is not its cause.

Adsorptive union of different proteins into a single complex as the basis for the "homogenization" process is eliminated by the experiments on heating the separate fractions and the experiments with mixing of previously heated albumin and globulin.

The similarity in electrophoretic properties of different denatured proteins is evidently caused by a break-up in their native specific structures coupled with the essential nature of denaturation.

The electrophoretic curves of native blood serum reflect the specific distribution of concentration of proteins as a function of their mobility, which determines the physiological relationships in the organism. When changes due to denaturation occur in proteins, we must assume a transformation from a specific distribution of properties (such, for example, as mobility in an electrical field) to a random distribution. It follows statistically that proteins with the mobility of albumin and γ-globulin should be present in very low concentration, since albumin has the greatest mobility and γ-globulin the least in serum. In fact these two proteins constitute the chief mass of blood serum proteins (75-80%). Denaturation is the transformation to the most probable state (increase of entropy) and therefore the normal replacement of an "improbable state," such as the high concentration of proteins with very characteristic mobilities, by a "probable" state characterized by a high concentration of protein structures with average mobilities.

What is the mechanism by which different protein fractions acquire about the same electrical charge? A consideration of the amino acid composition of blood serum proteins shows that the difference in the number of their basic and acid groups is small. The ratio of the number of acid amino acids (aspartic and glutamic acids) to basic (arginine, histidine, lysine) from the published data [26] is for albumin 1.33 and for γ-globulin, 1.48.

It is quite safe to assume that if instead of a specific structure of the protein chain there is formed a statistical globule, then albumin and γ-globulin will give electrophoretically similar forms. Gorbacheva, Bresler, and Frenkel' [27] from an ultracentrifuge study of the denaturation of horse serum albumin have concluded that the essence of the denatured modification is the transformation of the protein into a simple polyelectrolyte. It is natural to assume that the same process occurs in the denaturation of γ-globulin and so the similar ratio of acid and basic amino acids makes possible the appearance of electrophoretically similar forms. It is known that the calculations of the mobility of proteins as polyelectrolytes are very complicated and therefore more definite conclusions at this stage of the work cannot be drawn.

The general nature of the phenomenon of "electrophoretic homogenization" for different native protein mixtures is evidently an indication of the very general nature of the biosynthesis of albumins and globulins (in a definite biological group). It must be assumed that the amounts and types of amino acids in albumin and γ-globulin of blood serum are widely similar and so "electrophoretic homogenization" is characteristic for different proteins.

As to the term "electrophoretic homogenization" itself, it is possible that it corresponds to an actual homogenization, if by the latter is understood the appearance of a group of modified proteins whose mobilities vary with small dispersion.

This effect of "electrophoretic homogenization" has a direct relation to the problem of the origin of α- and β-globulins [7,9]. An effect closely related to the origin of α- and β-globulins and outwardly very similar to "electrophoretic homogenization" was called by us "α, β-globulinization" [7]. If we could finally show the identity of these two effects, then for serum we should replace the term "electrophoretic homogenization" by the term "α, β-globulinization" which would more accurately reflect the true situation.

The question of the formation of globulin from albumin has been in the literature for a long time (Mikhailov, 1888). However, the opinion has been established that such a process is impossible without deep rearrangement, because of the difference in amino acid composition of these two proteins [22]. It should be mentioned that an objection based on study of amino acid composition can hardly serve as an argument, since it is known that the compositions of α- and β-globulins are those of different proteins. If parts of them are modified forms of albumin and γ-globulin then only in these two can the true relationship of the amino acids be sought.

SUMMARY

We have established a single type of electrophoretic change in a series of animal and plant proteins after denaturing by heat or urea, shown by a tendency toward increase in the content of one fraction. The mobility of this fraction is close to the average mobility of the proteins in a given mixture in most cases. Heating in a strongly alkaline medium (pH 11.0) gives full "homogenization," that is, all the proteins of a mixture are transformed to give one electrophoretic peak.

When the separate fractions of blood serum proteins are heated it is found that the γ-globulin gives a fraction with greater mobility in an electric field, and albumin gives one with less. These results, like those with artificial mixtures, show that "electrophoretic homogenization" is due to the properties of the separate proteins and not to their mixture. The basic reason for "electrophoretic homogenization" is not aggregation nor the formation of adsorption complexes.

The basis of this effect must be transformation from a specifically organized native protein to a chaotic, denatured state which produces a statistically average set of properties for the protein, especially for the mobility. Probably the similarity in the amino acid composition of blood proteins gives a statistical distribution for mobility with little dispersion, which produces the visible homogenization.

We emphasize the relation of the "electrophoretic homogenization" with the formation of α- and β--globulins in animal blood.

Received December 4, 1957

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ON THE PROPERTIES OF LIPASE FROM OIL-BEARING PLANTS

A. A. Prokof'ev and G. V. Novitskaia

Institute of Plant Physiology of the Academy of Sciences SSSR, Moscow

As is well known, lipases of animal origin are divided into two groups on the basis of substrate specificity. One group is represented by the liver enzyme which splits esters of the lower fatty acids, while the other group, represented by pancreatic lipase, splits glycerides of the higher fatty acids. With regard to lipases of plant origin, the question of substrate specificity cannot be considered entirely solved.

The lipase from the seeds of the castor plant [1] and of rape [2], and from certain mold fungi [3] are the most thoroughly studied. Concerning lipases from other plant material, there is only isolated, fragmentary information [4].

On the basis of materials obtained with the castor plant lipase, the supposition arose that lipase has a universal activity extending to complex esters from extremely varied sources. Thus, for example, the castor plant lipase is effective in splitting castor, olive, linseed, rape, almond, soy, cottonseed or peanut oils and also bone marrow fat and fish oil. Along with this, castor plant lipase also splits the complex esters formed by monohydroxy alcohols (ethyl, methyl, amyl) and, for instance, such acids as acetic, butyric, malonic, and also benzoic and salicylic. Cholesterol esters are not split by the castor plant lipase [5].

The main problem of the present work was a clarification of the question concerning the presence of substrate specificity in lipases from oil-bearing plants: poppy, flax and sunflower.

METHODS

For determining lipase activities of ripening seeds, poppy, flax and sunflower plants were grown during the 1956-1957 vegetation period at the experimental division of the Institute in Moscow.

The following were taken for determinations of activity in dormant and germinating seeds: poppy seeds from the 1956 oil crop, flax from the 1955 Bukhara crop, and sunflower seeds from the 1956 Krasnodarsk 8931 (super-elite) crop.

Lipase activity was determined by titrating the fatty acids formed during the experiment with a 0.1 N solution of NaOH.

In order to determine the dependence of the lipase activity on the pH of the medium, 2.5 g of seed was ground in a mortar with 15 ml of water and then transferred to 100-ml flasks to which were added 10 ml of buffer, 2 ml of oil (corresponding to the character of the seeds — poppy, linseed or sunflower oil) and several drops of toluol. The enzyme in control flasks was inactived by boiling for 5 minutes. Experimental and control flasks were placed on a shaker for 48 hours at 37°, after which 10 ml of ethyl ether and 40 ml of alcohol were added. Titrations were carried out with 0.1 N NaOH with thymolphthalein. Lipase activity was determined by the difference in amount of base used in titrating control and experimental samples (in ml of 0.1 N NaOH). For a pH of 3-4, McIlwain's phosphate-citrate buffer mixture was used, Sorensen's phosphate mixture for pH 5-8, and Sorensen's borate mixture for pH 9-10.

For determinations of substrate specificity of the lipase, poppy, flax and sunflower seeds were defatted by treating them with five volumes of petroleum ether (46-60°) not less than five times for 45 minutes each time. One g of defatted seed meal was used for each experiment. The dependence of lipase activity on the

pH of the medium in ripening poppy, flax, and sunflower seeds was determined at the time of the highest lipase activity (poppy – 20-21 days, flax – 20-26 days, sunflower – 20-23 days after start of blooming) [6,7].

RESULTS

In order to be able to compare the action of lipases from seeds of various plants, it was first necessary to determine the manner in which enzyme activity changes in connection with the state of the seeds and the pH conditions of the medium. For this purpose, the lipase activity of poppy, flax, and sunflower seeds was determined in the range of pH values from 3 to 10.

Dormant, germinating, and ripening seeds of the indicated oil-bearing plants were investigated. The results obtained are presented in Table 1.

TABLE 1

The Dependence of Lipase Activity of Poppy, Flax and Sunflower Seeds on the pH of the Medium

Lipase activity in ml of 0.1 N NaOH per 1 g of absolutely dry weight

pH	Poppy			Flax			Sunflower		
	ripening	dormant	germinating	ripening	dormant	germinating	ripening	dormant	germinating
3	12.2	12.3	1.5	9.6	15.6	2.1	5 3	5.6	2.8
4	18.2	19.5	4.8	14.8	19.5	7.5	5.6	5.9	3.9
5	23.8	6.2	15.2	17.8	12.4	11.0	17.7	1.2	4.6
6	14.8	6.0	4.7	3.5	4.1	9.6	5.4	1.1	4.3
7	16.2	6.3	4.0	3.8	3.0	8.0	11.4	1.2	3.7
8	23.5	11.0	3.4	8.3	5.8	5.7	12.6	4.9	3.5
9	23.6	3.7	2.9	8.1	2.5	5.7	9.4	1.6	2.8
10	22.5	3.6	2.7	8.1	1.6	3.2	8.8	0.9	2.6

As seen from Table 1, enzyme activity depends on the state of the seeds and the pH of the medium. The lipase of dormant and ripening seeds has two clearly demarcated pH optima, one of which falls in the acid region, and the other in the alkaline. The lipase of germinating seeds has only one optimum in the acid region. It should also be noted that in the acid region the optimum has a narrower range than in the alkaline where, in ripening seeds, for example, it covers a wide range (pH 8-10).

TABLE 2

Activity of Lipase From Poppy, Flax and Sunflower Seeds on Various Substrates

Substrate	Poppy		Flax		Sunflower	
	lipase activity in ml of 0.1 N NaOH					
	per 1 g of absolutely dry wt. of de-fatted meal	% of hydrolysis	per 1 g of absolutely dry wt. of de-fatted meal	% of hydrolysis	per 1 g of absolutely dry wt. of de-fatted meal	% of hydrolysis
Castor oil	2.5	8.9	5.6	16.8	12.1	36.5
Olive oil	3.8	11.2	7.1	20.8	16.4	47.1
Poppy-seed oil	5.6	16.2	6.6	19.3	13.8	45.0
Sunflower oil	3.4	9.9	7.8	22.8	17.5	50.9
Peanut oil	—	—	7.5	21.4	16.3	46.4
Mustard oil	3.1	9.8	8.5	27.3	6.8	53.9
Linseed oil	6.0	17.5	6.9	20.0	10.9	29.5
Ethylacetate	13.4	13.3	1.2	9.6	19.1	16.6
Butylacetate	12.0	13.1	5 6	6.0	6.7	7.3

Proceeding from the data obtained, we conducted investigations of substrate specificity at a pH optimal for the given material (pH 4 for dormant seeds).

Results of the determinations of substrate specificity of the lipases are given in Table 2.

As seen from Table 2, lipases from the seeds of all the plants investigated split the glycerides of various oils rather well. As for the esters of lower acids, they too were split by the lipase preparations; this can be taken as evidence either of the presence of other active esterases in the preparations used by us, or that the lipase possesses esterase activity with respect to these substrates.

An examination of the data given makes it reasonable to conclude that the lipase of the investigated plants splits the esters of the lower, as well as of the higher fatty acids and, consequently, it is not possible to speak of any clearly expressed specificity of this enzyme.

TABLE 3

Action of Various Substances on the Lipase Activity of Poppy, Flax and Sunflower Seeds

Substance	Concentration in M	Poppy		Flax		Sunflower	
		Lipase activity in ml of 0,1 N NaOH					
		per 1 g of absolutely dry wt. of seeds	in % of control	per 1 g of absolutely dry wt. of seeds	in % of control	per 1 g of absolutely dry wt. of seeds	in % of control
Glutathione	0.01	10.3	165	8.0	137	5.6	192
Cysteine	0.01	7.4	119	6.9	117	3.7	126
Ascorbic acid	0.01	13.0	210	8.8	150	6.2	211
Glycine	0.1	6.2	100	4.9	83	2.8	96
Na acetate	0.1	8.7	140	3.7	63	3.3	111
$CaCl_2 \cdot 2H_2O$	0.1	6.0	96	1.9	33	2.2	74
KCN	0.1	1.2	19	1.1	19	0.9	33
NaF	0.1	5.6	89	3.6	61	2.7	92
Resorcin	0.1	6.4	103	5.4	93	3.8	129
Control		6.2	100	6.9	100	2.9	100

It was of interest to determine the relationships of the lipases of various plants to a number of substances known as activators and inhibitors of lipase activity.

Glutathione, cysteine, ascorbic acid, Na acetate, $CaCl_2 \cdot 2H_2O$, KCN, NaF, and resorcin were tested. Samples lacking the indicated substances served as controls.

The results of the experiments are presented in Table 3.

As can be seen from the data in Table 3, glutathione, cysteine, and especially ascorbic acid showed an activating effect. KCN and NaF, on the contrary, inhibited lipase activity – potassium cyanide to a greater degree than NaF. Although most authors consider calcium to be an activator of lipase activity, it acted as an inhibitor in our experiments at the given concentration. Comparison of the data on activation and inhibition of the lipases from various plant seeds gives reason for speaking of the similarities, rather than of the differences of the enzymes.

SUMMARY

Lipase activity depends on the physiological state of the seeds, and also on changes in the pH of the medium. The lipases of dormant and ripening poppy, flax, and sunflower seeds have two pH optima (4-5 and 8-10), while the lipases of germinating seeds have one (pH 5).

Fairly good splitting of the glycerides of various oils by lipase preparations from poppy, flax, and sunflower seeds indicates an absence of substrate specificity for the lipases of the plants investigated. The decomposition of ethyl acetate and butyl acetate is evidence either of the presence of other active esterases in the seeds of the indicated plants, or of the ability of the lipase to split the esters of lower acids.

Glutathione, cysteine and ascorbic acid had a marked activating effect on the lipases of the indicated plants, while KCN and NaF inhibited their activities.

Received December 10, 1957

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A STUDY OF THE PROPERTIES OF PHOSPHOGLUCOMUTASE OF POTATO TUBER EXTRACTS AND IN ENZYME PREPARATIONS ISOLATED FROM THEM

T. T. Bolotina

Laboratory of Physiological Chemistry, Academy of Sciences, USSR, Moscow

As our investigations have shown [1-5] phosphoglucomutase (PGM) plays a decisive role in the formation of starch in potato tubers. Depending on the intensity of transformation of glucose-1-phosphate (G-1-P) to glucose-6-phosphate (G-6-P), which is the reaction catalyzed by phosphoglucomutase, some amount of glucose-1-phosphate can be removed from the sphere of action of the phosphorylase. Thus, the synthesis of polysaccharides is closely connected not only with the activity of phosphorylase, but also with the action of phosphoglucomutase, enzymes which attack the same substrate.

The present work, a continuation of the investigations published earlier [1-5], gives the results of a study of the properties of the phosphoglucomutase from potato tubers in extracts and in preparations of the enzyme isolated from them.

METHODS

A water extract of the tubers was prepared, and the phosphoglucomutase activity was determined by the methods described earlier [6, 1]. The preparation of PGM was isolated from the aqueous extract by two methods.

1. Fractionation with ammonium sulfate gave the 0.4-0.6 saturated fraction which was once reprecipitated. After dissolving the precipitate and dialysis, we determined the activity of the resulting preparation.

2. The fraction of 0.4-0.6 saturated $(NH_4)_2SO_4$ was dissolved and after cooling it to 0°, the phosphorylase was removed by adsorption on crystals of NH_4MgPO_4 [7], for which the solution was treated with NaH_2PO_4 and $MgSO_4$ and the pH of the mixture brought to 8.0 with a solution of ammonia. After standing 2 hours at 0° the solution was filtered and PGM was precipitated from the filtrate with ammonium sulfate.

EXPERIMENTAL PART

Study of the Optimal Conditions for the Action of Phosphoglucomutase from Potato Tubers in the Extract

From the data given in Table 1 it follows that the maximum activity of PGM is shown when 3 ml of incubation mixture contains 1.5 ml of extract. With decreased amount of extract the rate of the phosphoglucomutase reaction is slowed. Further, the equilibrium state of the PGM reaction is reached faster, the less substrate there is the incubation mixture.

Activation of PGM in the extract is brought about by Mg ions and reducing agents, sodium sulfite [2]. As Table 1 shows, the maximum activity of phosphoglucomutase is found at magnesium sulfate concentrations of 1.25 mg/ml and sodium sulfite concentrations of 2.5 mg/ml of incubation mixture. At concentrations of the activators above the optimum we note an inhibiting action on the enzyme, and lower concentrations are insufficient for full activation of PGM.

As our experiments show (Fig. 1) the maximum activity of phosphoglucomutase is found at pH 7.5. At more alkaline pH values the reaction rate is slowed. The shift of pH to the acid side also acts unfavorably on the PGM reaction; there is a sharp fall in activity of the enzyme.

TABLE 1

Effect of Different Ingredients of the Incubation Mixture on the Rate of the Phosphoglucomutase Reaction in the Extracts

Incubation mixture: extract + glucose-1-phosphate + 0.1 N veronal buffer (1 ml) + Na_2SO_3 + $MgSO_4$ + H_2O. Total volume 3 ml. Amount of G-1-P transformed into G-6-P expressed in % of starting value

Amount of substance taken		Incubation time in min			Amount of substance taken		Incubation time in min		
		0	30	120			0	30	120
Extract in ml	1.5	0	74	80	Magnesium sulfate in mg/ml of incubation mixture	0.3	0	34	61
	1.0	0	66	88		0.6	0	46	84
	0.5	0	30	95		1.2	0	71	95
	0.25	0	2	50		2.5	0	58	90
						5	0	29	65
Glucose-1-phosphate in mg/ml of incubation mixture	3	0	95	95					
	5	0	86	93	Sodium sulfite in mg/ml of incubation mixture	0.6	0	37	66
	7	0	80	93		1.2	0	51	81
	9	0	47	87		2.5	0	62	84
	11	0	31	86		5	0	40	80
						10	0	16	55

Note: In each variant of the incubation mixture the content of one component was changed; all the other components were added in optimum amount.

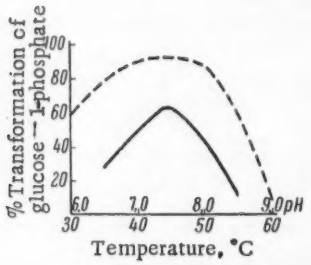

Fig. 1. Effect of temperature and pH of the incubation mixture on rate of phosphoglucomutase reaction in the extract.
– – – Temperature; —— pH.

PGM has a high activity in the temperature interval 40-50° (Fig. 1). Increase in temperature to 60° inactivates the enzyme completely. At lower temperatures the rate of the reaction decreases.

Study of the Optimum Conditions of Action of Phosphoglucomutase Preparations Isolated from the Extracts

Our study showed (Table 2) that the equilibrium state of the reaction is reached faster when there is more enzyme preparation in the incubation mixture.

The optimum amount of substrate for this enzyme is 7 mg/ml of incubation mixture; with increasing amounts of substrate the reaction rate is slowed. Amounts of substrate below the optimum act unfavorably on the PGM reaction; in this case the reaction reaches a lower limit.

In studying the effect of different amounts of magnesium sulfate on the rate of the PGM reaction, we found the same relation as in the extract.

The PGM preparation is inactive in the absence of a reducing agent.

When PGM is activated by sodium sulfite we find the same relation as in the extracts.

The sodium salt of cysteine also activates PGM. The optimum concentration of this compound is 6 mg/ml of incubation mixture. Concentrations below the optimum are insufficient for full activation of the enzyme, and concentrations above the optimum destroy the activity to some extent. Preparations of PGM and PGM in the extract have the same optimum pH.

In the temperature interval 40-50° the enzyme activity is high enough, just as in the extract, but in experiments with enzyme preparations the optimum temperature is shown more clearly; the maximum reaction rate is found at 45° (Fig. 2).

TABLE 2

Effect of Different Ingredients of the Incubation Mixture on Rate of the Phosphoglucomutase Reaction

Incubation mixture: enzyme preparation + glucose-1-phosphate + veronal buffer + $+Na_2SO_3 + MgSO_4 + H_2O$. Total volume 3 ml

Amount of substance taken		% Transformation of glucose-1-phosphate				
		0 min	15 min	30 min	60 min	120 min
Enzyme preparation in ml	1.0	0	27	95	95	—
	0.5	0	40	95	95	—
	0.25	0	20	50	95	—
Glucose-1-phosphate in mg/ml of incubation mixture	3	0	—	—	74	74
	5	0	—	—	75	78
	7	0	—	—	61	80
	9	0	—	—	43	77
	11	0	—	—	39	70
Magnesium sulfate in mg/ml of incubation mixture	0.3	0	22	69	—	
	0.6	0	26	90	—	—
	1.2	0	28	96	—	—
	2.5	0	17	66	—	
	5	0	0	12	—	
						—
Sodium sulfite in mg/ml of incubation mixture	0	0	—	0	0	—
	0.6	0	—	15	35	—
	1.2	0	—	26	52	—
	2.5	0	—	36	83	—
	5	0	—	30	75	—
	10	0	—	24	43	—
						—
Cysteine in mg/ml of incubation mixture	0	0	—	0	0	—
	2	0	—	22	36	—
	4	0	—	24	53	—
	6	0	—	41	70	—
	8	0	—	33	70	—
						—

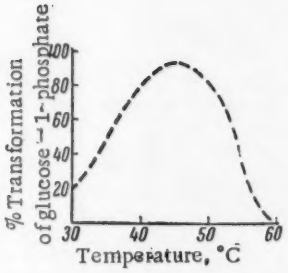

Fig. 2. Effect of temperature of incubation mixture on rate of phosphoglucomutase reaction with enzyme preparation.

DISCUSSION OF RESULTS

The study of the optimum conditions for action of phosphoglucomutase in extracts and in preparations isolated from them by fractionation with ammonium sulfate showed that the properties agreed in a number of cases: the same optimum pH, the same optimum concentration of activators, etc.

However, there were some differences in behavior of PGM in extracts and in isolated preparations. Thus, in the experiments with the preparations we found a more sharply expressed temperature optimum; in studies of the effect of amount of extract on rate of the PGM reaction we found that the limits reached by the reaction were lower than those in the experiments with the preparation. These differences in behavior of the enzyme in the extracts and in the isolated preparations could probably be explained by the presence in the extracts of another enzyme (phosphorylase) and different conditions in the medium.

SUMMARY

We have studied the optimum conditions of action of phosphoglucomutase from potato tubers in extracts and in preparations isolated by fractionation with ammonium sulfate.

I express thanks to Doctor of Biological Science A. N. Petrova for valuable advice in carrying out this work.

Received December 12, 1957

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HEXOKINASE FORMATION IN MOUSE ASCITIC CANCER CELLS

T. B. Kazakova

Biochemistry Department, The Institute of Experimental Medicine,
Academy of Medical Sciences, Leningrad

The metabolism of the cancer cell is characterized by a very high glycolysis level, and this fact, together with the pronounced Pasteur effect in these cells, has continuously prompted investigations of the various glycolytic enzymes in tumors.

Interesting results in this field were obtained by Shade [1], who used ascites, which are vigorously growing suspensions of homogeneous and separate cells within the ascitic fluid of animals. This author found that, when these tumor cells were incubated in vitro in the ascitic fluid, there took place a considerable increase in the activity of various enzymes of glycolysis, namely, aldolase, triosephosphate dehydrogenase and triosephosphate isomerase.

It was established somewhat earlier by Warburg [2] that blood aldolase activity was rather high in rats with Jensen's sarcoma, and that this activity tended to increase further with the development of the tumor. The same phenomenon was demonstrated by the Hungarian workers [3] for the serum adenosine deaminase, in mice bearing Ehrlich ascites, and by Hill and Yordan [4], in the case of lactic dehydrogenase in the blood of cancer-bearing mice.

It is of interest to note that Neiman [5] found increased levels of glycolysis in cancer-bearing animals even in organs which were unaffected by the growth.

A consideration of all these data suggested the possibility that the glycolytic enzymes were continuously synthesized within the cancer cell, whence they were passed into the ascitic fluid and, subsequently, into the blood stream of the tumor-bearing animal.

Hexokinase (HK) occupied a special position among the glycolytic enzymes. Neifakh, Mel'nikova and Fomina [6, 7] showed that the over-all glycolytic rate depended on the "rate-determining enzymatic activity" in the glycolytic chain. According to these authors, this enzyme in the tumor cells was hexokinase.

The question thus arose, was not hexokinase synthesis within the cancer cell responsible for the observed high level of glycolysis, and, if this were true, would it not be possible to demonstrate HK synthesis in the tumor tissue within a comparatively short period.

The present communication deals with the dynamics of HK formation in mice bearing Ehrlich ascites, as determined by the assay of HK activity in the tumor cells themselves, in the ascitic fluid and in the plasma.

METHODS

Ehrlich ascites transplantations were made by the intraperitoneal introduction of 0.3 ml of a 6-7-day-old ascites suspension to mice weighing 15-20 g.

Investigations of the ascites suspension, carried out for the purpose of strain characterization, showed that the cells reached maximal density (dry weight, mg/ml of washed cells) and maximal ribonucleic acid content per cell, 6-8 days after inoculation, which indicated that the cells had reached full maturity at that time. The highest rate of tumor growth coincided with this period. This rate decreased thereafter, the number of dead cells becoming increasingly higher, and the animals usually died on the 16th day (Fig. 1).

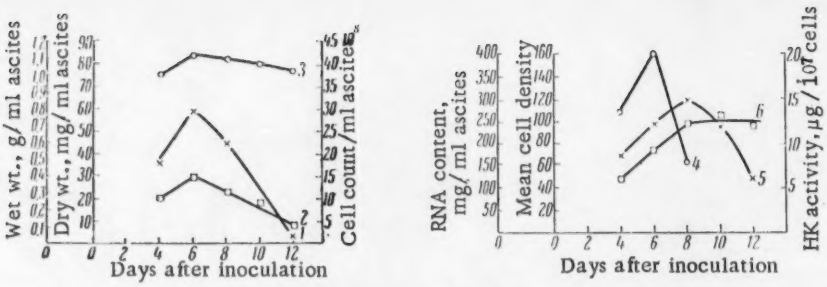

Fig. 1. Culture characteristics of the strain of Ehrlich ascites carcinoma used. 1) Cell count; 2) dry weight (of cell); 3) wet weight; 4) RNA content; 5) mean cell density; 6) HK activity.

Dry weights were determined by drying at 105°, cell counts were carried out in a Goriaev chamber, and packed cell volume was determined by centrifuging 1 ml of the ascites suspension in a graduated tube for 10 min at 1000 rpm.

All HK assays were thereafter carried out on ascites suspensions harvested on the 6-7th day of the tumor growth.

When HK activity was assayed in the later stages of the tumor development, the calculated mean activity per cell remained approximately constant, since the number of cells decreased, while HK activity in the ascitic fluid and plasma increased.

Ascites suspension was harvested from four animals, heparinized and mixed thoroughly (cell count), and the pooled suspension divided into equal parts. One portion was assayed immediately for HK activity (initial value), while the other was incubated under conditions which varied in different experiments, HK activity being determined in this suspension at the end of the incubation period (final value). The difference between the HK values in the initial and final sample gave an indication of HK neosynthesis in the sample.

For hexokinase assays ascites suspensions were centrifuged, the ascitic fluid was removed, and the cell pellet washed with 10 volumes of 0.15 M KCl. The cells were then suspended in 0.15 M KCl, and the suspension used in HK assays.

Hexokinase assays were carried out in two ways, namely, using the commonly employed disrupted cell suspensions, and in suspensions of intact cells. The latter method was found to be more convenient, since hexokinase was found to be present on the surface of the ascitic cells, as an active ectoenzyme. The presence of $5\text{-}9 \cdot 10^7$ cells in a single test was sufficient to demonstrate the surface HK. Cell extracts were produced using either a glass Potter-Elvehjem homogenizer with a minimal clearance, at 200 rpm, or a therapeutic ultrasonic apparatus (supplied by VEM, Germany), at a frequency of 800 kc, power output 4 w/cm^2, for 10 min. Cell disruption in each case reached 80%, which was assessed by cell counts in the Goriaev chamber and confirmed by failure to transplant. The homogenates (or sonicates) were centrifuged at 2000 rpm. The pellet was discarded and the supernate was used for assays. Protein content was determined by the biuret method, and ranged from 1.5 to 2.0 mg per aliquot.

HK activity was assayed by the disappearance of glucose, essentially according to Long [8], but the incubation mixture was altered in composition to suit the kinetics of the tumor enzyme, and was as follows (final concentrations, M):

Potassium phosphate buffer, pH 7.8	0.042
$MgCl_2$	0.006
Glucose	0.0039
Extract	1.5-2.0 mg protein
NaF	0.06
K-ATP	0.004-0.005

Incubation time, 15 min at 37°.

In view of the possibility of reducing matter arising during cell incubation in vitro [9], which would lead to artificially decreased HK assays, control incubations were carried out in order to determine the total content of reducing compounds before and after incubation.

EXPERIMENTAL

HK Formation in Tumor Cells

Concurrent with HK assays in the extracts described above, attempts were made to assay HK also in intact tumor cells. Glucose disappearance was, in fact, observed when intact ascites were incubated under the conditions described for extracts above. This activity was only observed in the presence of the complete incubation mixture, and did not take place if any single component of the system was absent.

The mean value of HK activity, as determined by this method and expressed as Q_{HK} (µl of gas/mg of dry tissue/hour), was found to be 15 µl/min before incubation, and 26 µl/min after.

HK activity of the whole cells was thus found to be 30% lower than the corresponding activity of the cell extract (Q_{HK} found – 20 µl/mg and 34 µl/mg before and after incubation, respectively).

The comparatively lower HK activity of the whole cells was due to the fact that only a portion of the total enzymatic activity, namely the surface ectoenzyme, was assayed in the whole cell. Previous investigations [10] have revealed that HK activity of tumor cells was higher than that of the normal counterparts, and that, within the tumor cell, this activity was concentrated mainly in the mitochondria [11]. It is now clear from the present data that the surface of the tumor cell also contains active HK. This is hardly surprising in view of the known fact that glucose, which is the main nutrient of the tumor cell, can only enter it through the mediation of hexokinase [12]. The presence of ATPase as an ectoenzyme on the surface of ascites [13] and of the nucleated erythrocyte [14] has been described previously.

In the study of HK formation in ascites suspension the natural medium – ascitic fluid – was used in incubations.

Simultaneous incubations of ascites suspensions were carried out in 0.15 M KCl at 37°, and in ascitic fluid at 0° and at 37°. The fluid was removed after the incubation and the cells were washed in 0.15 M KCl; HK was then assayed in the whole or disrupted cells (extract). "Initial" HK activity was previously assayed on freshly harvested cells, similarly treated.

It will be seen from Table 1 that no difference between the "initial" and "final" HK activity values, and therefore no HK neosynthesis, was observed in cells incubated either in KCl, or in ascitic fluid at 0°. On the other hand, a pronounced increase in HK activity, whether assayed on whole or disrupted cells, was observed when the cells were incubated in ascitic fluid at 37°.

TABLE 1

The Change in HK Activity During the Incubation of Tumor Cell Suspensions

HK activity has been expressed as µmoles of glucose phosphorylated in 15 min per 1 mg protein

Enzyme source	non-incubated cells	cells incubated in KCl at 37°	cells incubated in ascitic fluid		Enzyme source	non-incubated cells	cells incubated in KCl at 37°	cells incubated in ascitic fluid	
			0°	37°				0°	37°
whole cells	25	24	25	47	extract	24	25	26	54
	26	30	26	43		32	34	37	44
	27	27	25	44		41	40	42	58
	27	25	27	56		62	56	60	86
	36	36	37	58		62	68	54	74
	47	47	46	67		64	–	–	125

Time-activity HK assays showed that the activity increased during the incubation, reaching a maximal value at 45 min, after which period the activity fell, apparently as a result of damage to the cellular enzymatic systems (Fig. 2).

In order to establish any relation between the fluctuations of HK activity and the state of aerobiosis or anaerobiosis, cell suspensions were incubated, in different experiments, in 95% O_2 + 5% CO_2, and in 95% N_2 + 5% CO_2. The incubations were carried out in Warburg vessels, for varying time intervals, at 37°.

It will be seen from Fig. 3 that, although the activity increased under both aerobic and anaerobic conditions, the rates of increase were different in the two instances. Thus, under aerobic conditions the activity reached 150% of initial value in 30 min, while under anaerobic conditions this was only 110%.

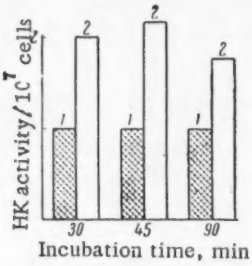

Fig. 2. The change in HK activity in tumor cells incubated in air. The data represent a mean of 7 experiments. In this, and in all subsequent figures HK activity of nonincubated cells will be taken as unity. 1) Nonincubated cells; 2) incubated cells.

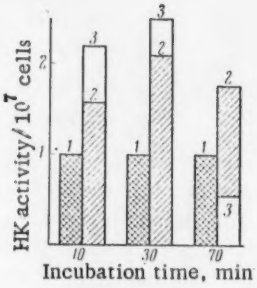

Fig. 3. The change in HK activity of the tumor cells during incubation in atmospheres of O_2 + CO_2 and N_2 + CO_2. The data represent means of 7 experiments. 1) Nonincubated cells; 2) cells incubated under N_2 + CO_2; 3) cells incubated under O_2 + CO_2.

Several explanations could be offered for this phenomenon in the initial stages of the work. First, it could be assumed that the apparent increase in activity, as observed in the intact cells, was not due to increased enzyme synthesis, but to a transfer of intracellular HK to the external surface. Secondly, there may have been some liberation from the cells of a HK activator during the incubation in the ascitic fluid. Thirdly, true enzyme formation could have taken place through the intracellular synthesis of HK molecules.

The possibility of HK transfer from within the cell to the outer surface was excluded in the following experiments. HK activity was assayed on whole cell homogenates of both freshly harvested and incubated cells. If intracellular enzyme transfer to the surface was the only phenomenon during the incubation, the homogenates of the two groups could be expected to possess equal activity. It was found, however, that incubation resulted in a 70-100% increase in HK activity.

The possibility of release of activators did not lend itself to direct investigation, and therefore indirect approaches were tried in this case. Thus, cells were preincubated and homogenized, and the homogenates were mixed with suspensions of freshly harvested cells. The HK activity of the fresh suspension and of the homogenate were assayed separately, as well as the activity in the mixture.

A release of an activator during the incubation would result in further activation of the HK in the nonincubated cells on mixing with the incubated homogenate. The total activity in the mixture would therefore be expected to be greater than $(A+B)/2$, where A and B are the respective activities of the incubated and nonincubated suspension. This was not the case in practice.

As seen from Fig. 4, the activity in the mixture was found to be equal to the arithmetical mean of the activities of its two components. This observation was taken as evidence against the formation or release of a HK activator during the incubation.

In all subsequent experiments the assumption was made that, if the increase in HK activity depended on factors and conditions known to be favorable for the synthesis of other proteins, the actual synthesis of HK was a more likely event than the formation of activators.

The energetic requirements for the phenomenon were investigated first. This was carried out through the use of various poisons which inhibit phosphorylation. No increase of HK activity could be observed when the cells were incubated in the presence of $2 \cdot 10^{-4}$ M 2,4-dinitrophenol (DNP), which is known to cause the uncoupling of phosphorylation from oxidation. (Fig. 5).

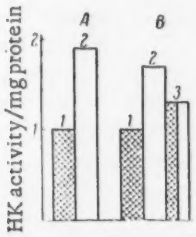

Fig. 4. HK activity after mixing incubated and nonincubated cells. A) Mean of 21 experiments; B) mean of 6 experiments. 1) Nonincubated cells; 2) cells incubated 30 min; 3) mixture: incubated + nonincubated.

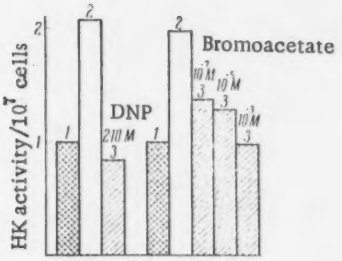

Fig. 5. The effect of DNP and bromoacetate on the increase in HK activity during the in vitro incubation of tumor cells. 1) Nonincubated cells; 2) cells incubated 30 min; 3) cells incubated 30 min in the presence of inhibitor shown.

This result was in good accordance with our data on the predominantly aerobic character of this phenomenon.

There exists published opinion [15] to the effect that DNP should have little influence on the over-all phosphorylation rate in tumor tissues, since any inhibition of oxidative phosphorylation would be compensated for by an equivalent adjustment of glycolytic phosphorylation levels. However, if it be assumed, despite existing data [16], that this held for phosphorylation rates, the fact still remained that DNP was found to exert pronounced inhibition on protein synthesis in Ehrlich ascites carcinoma [17].

This finding was confirmed by the present results. The complete inhibition found in the present work was apparently due to the high ($2 \cdot 10^{-4}$ M) DNP concentration, which was one order of magnitude higher than the concentrations ($2 \cdot 10^{-5}$ M), at which the uncoupling effect has been observed in ascites cells [18]. It is of interest that Hokin [19] found DNP, in concentration $2 \cdot 10^{-4}$ M, to exert a 99% inhibition on amylase synthesis in pigeon pancreas slices, regardless of the fact that this synthesis took place anaerobically and exclusively at the expense of glycolytic phosphorylation. It would appear that the DNP effect encompasses not only the process of oxidative phosphorylation, but also mechanisms of protein synthesis.

Further experiments were carried out with the use of bromoacetate; this inhibitor is known to interrupt glycolysis and, at high concentration, to interfere with a number of other thiol enzymes.

It will be seen from Fig. 5 that the phenomenon under investigation [HK neosynthesis] was inhibited by bromoacetate, and that the inhibition increased with increasing bromoacetate concentration, up to complete suppression of the phenomenon.

The results were therefore taken as an indication that the observed increase of HK activity was indirectly connected with the processes involving high energy phosphate bond regeneration and their utilization in protein synthesis.

It is now generally accepted [20-21] that ribonucleic acid (RNA) in a highly polymerized form is an obligatory component of any protein synthetic system, including that of tumors. This theory has received partial proof from the observation [22] that ribonuclease, which depolymerizes RNA, stops protein synthesis. Ribonuclease action may be demonstrated not only in solution, but also in cell suspensions. Thus Ledoux [23] showed that RNA-ase, added to ascites cell suspensions, broke down the cytoplasmic RNA within 20 min.

It was therefore of interest to determine whether the increase in HK activity, observed in the present case, depended on the polymerized state of the ascites RNA, that is, on the factor which is obligatory for protein synthesis.

Cell suspension aliquots were incubated as usual, with the exception that 6 units of crystalline RNA-ase (in solution, containing 100 μg protein per ml) was added to each aliquot.

It may be seen from Fig. 6 that in the presence of RNA-ase the increase in HK activity was completely abolished.

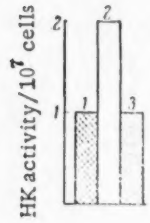

Fig. 6. The effect of RNA-ase on the increase of HK activity in tumor cells incubated in vitro. 1) Nonincubated cells; 2) cells incubated for 30 min; 3) cells incubated for 30 min in the presence of RNA-ase.

Since the abolition of increased HK activity occurred as a result of the rather mild and completely specific action of RNA-ase, there remained little doubt about the participation of protein synthesis processes in the observed increase in HK activity. Nevertheless, a further experimental series, employing folic acid antimetabolites, was carried out. Folic acid is known to be involved in nucleic acid synthesis, and could therefore be expected to influence protein synthesis also [24].

The interference with enzyme synthesis in vitro, and the inhibition of cell growth, in the presence of folic acid antimetabolites has been reported [25]. The protein synthesis-inhibiting agents employed were aminopterin and a home-produced antimetabolite – 4-aminopteroylaminoadipic acid (AD), synthesized by the chemical laboratory IEPITR, USSR Academy of Medical Sciences. The use of these inhibitors in the present work was also of a special interest in view of their wide application to oncological practice in the therapy of malignant tumors and acute leucocytosis [26].

In accordance with our expectations, both antimetabolites abolished the HK formation, AD being active at lower concentrations than aminopterin (see Fig. 7).

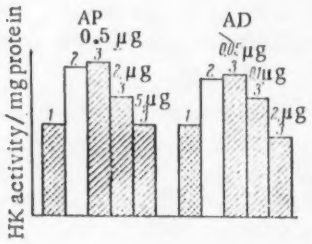

Fig. 7. The effect of aminopterin (AP) and of 4-aminopteroylaminoadipic acid (AD) on the increase of HK activity in tumor cells incubated in vitro. 1) Nonincubated cells; 2) cells incubated 30 min; 3) cells incubated 30 min in the presence of inhibitor shown.

HK Activity in Serum and Ascitic Fluid

While no in vitro experiments can categorically exclude the possibility of HK activators arising during incubations, there is no possible interpretation, on the activator basis, for the increase in enzymatic activity levels outside the tissue studied, in other parts of the body. In view of this the experimental work was shifted to the investigation of HK activity in the ascitic fluid and in the serum of the tumor-bearing mice.

The enzymatic activity in the mice ascitic fluid was initially assayed at various intervals after tumor inoculation. The HK activity in the fluid was found to be considerably lower than in the cells themselves, but, as the tumor progressed, the HK activity in the ascitic fluid increased. Thus, while the HK activity in the ascitic fluid, on the 6th day of tumor growth, was 0.006 μmole of glucose preformed in 15 min per mg protein, the value increased to 0.22 μmole on the 15th day, a 36-fold increase. However, this rather steep increase was possibly due not to the

passage of the enzyme from the cells to the fluid, but, in view of the advanced stage in tumor development, to the spontaneous disruption of the cancer cells themselves. Subsequent experiments therefore involved short term incubation of the cell suspension in the vessels.

After the incubation period the fluid was separated from the cells and its HK activity assayed.

The following results were obtained (activity expressed as μmoles of glucose phosphorylated at 37°, in 15 min, per 1 mg protein):

Nonincubated fluid	30-min incubation	18-hour incubation	24-hour incubation
0.006	0.02	0.03	0.005

It will be seen from the results that during the incubation of the cellular suspension, under exclusion of any possibility of enzyme elimination, the activity in the fluid increased steeply, particularly in the first 30 minutes. The increase in activity in the present case could hardly be explained by cell death, since cell lysis did not commence at 30 min. It was actually found that even after 24 hours only a small proportion of the cells died, inasmuch as subsequent transplantation resulted in positive takes, and it is known that, in order to obtain successful transplantations of the Ehrlich ascites carcinoma, the number of cells in the inoculum should be of the order of $1.5\text{-}2 \cdot 10^7$. The fall in activity after 24-hour incubation must be explained by enzyme inactivation.

The data quoted above confirm therefore the supposition that the enzyme actually passed from the cells into the surrounding fluid. Such transition of an enzyme from the cells into the fluid cannot be detected on the whole animal generally, probably because of the concomitant HK elimination from the peritoneal cavity of the mouse.

The transition of HK from the tumor cells into other tissues of the body can be demonstrated successfully by the assay of this enzyme in the serum. Assays were carried out on normal mice and on tumor-bearing mice, on the 7th day after transplantation.

It will be seen from Table 2 that HK levels in the serum of cancer-bearing mice were five times those of normals.

TABLE 2

HK Activity in the Serum of Normal and Tumor-Bearing Mice

The activity has been expressed as μmoles of glucose phosphorylated in 15 min per 1 mg protein

Normal mice	Tumor-bearing mice
0.0040	0.017
0.0040	0.019
0.0043	0.022
0.0043	0.018
0.0028	0.019
0.0043	0.019
0.0047	0.022
Mean value 0.004	0.02

Considering the appreciable degree of dilution in blood, the difference observed was rather large. It would appear obvious that this increase

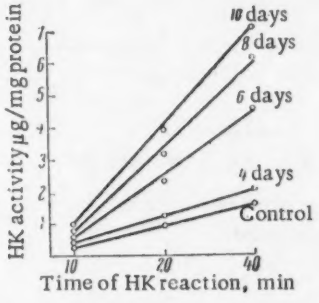

Fig. 8. The change in HK activity in the serum of tumor-bearing mice during tumor development.

in activity could not have occurred suddenly, but was an end result of a gradual process. This was actually demonstrated by following the daily levels of HK in the serum of mice from the inoculation onwards.

It will be seen from Fig. 8 that the enzymatic activity levels in the serum increased steadily with the tumor growth.

The situation therefore probably represents the continuous elimination of the enzyme from the peritoneal cavity, from the time of inoculation, followed by its transportation and distribution throughout the animal's body.

DISCUSSION

Increase in enzymatic activity in an in vitro system may be taken as evidence of net protein synthesis only when such synthesis is achieved on model systems obtained from disrupted cells [27, 28].

Results obtained with whole cells, whose organization is intact, are always subject to objection with regard to the possible formation of activators and other favorable factors likely to boost the investigated catalytic activity, and thus tending to give the impression of the synthesis of the enzymatic protein. Even the varied and precise evidence of amylase synthesis in pigeon pancreatic slices, quoted by Hokin [19, 29], cannot be taken as being completely reliable for the above-mentioned reasons.

Nevertheless the present authors are inclined to believe that formation of enzymatic protein has been demonstrated in the present study of Ehrlich ascites carcinoma.

Since no information about the nature of the materials which served as precursors in the formation of the enzyme is presently available, it is impossible to state that protein synthesis has been domonstrated. However, due to the high rate of HK appearance and because of ascitic fluid accumulation, formation of enzymatic protein was fully demonstrated by means of the short-term incubation of the cell suspension and the finding of increased enzymatic activity not only in the cells themselves, but also in the ascitic fluid and, further, in the serum of the tumor-bearing animal. This phenomenon can only be demonstrated using malignant tumors, since this type of protein circulation cannot be observed in the normal organism.

It is seen from the results presented above that the increase in HK activity in the cells took place only under the set of conditions (temperature, natural incubation medium, aerobic conditions) which was most favorable for cell survival and, consequently, for their protein rejuvenation.

It was shown, on the other hand, that the various factors which suppress protein synthesis (phosphorylation-uncoupling agents, RNA-ase, folic acid analogs) also abolished the increase in HK activity.

The increase in HK was thus closely connected with protein synthesis. It is quite feasible that under the favorable conditions there took place the synthesis of a number of cellular proteins, including HK. The other possibility – the formation of a proteinaceous HK activator – seems to be most unlikely. However, the most convincing argument in favor of true HK synthesis is the observed steady increase, throughout the development of the tumor, of this enzymatic activity in the ascitic fluid and in the serum.

Only the continuing synthesis of the enzyme in the ascitic cells could cause its steady increase of activity in the body fluids in which it is never found normally.

It is clear that protein synthesis is not confined to HK alone. The appearance in the serum of a number of enzymes concerned in carbohydrate-phosphorus metabolism has been demonstrated [3, 30] under conditions where the growth of the tumor was not accompanied by cell lysis and, consequently, the tumor cells alone could serve as the enzyme source. The formation of HK is of particular interest since the over-all activity of this enzyme apparently determines the glycolytic level of a tumor [6].

The conditions for HK formation in tumor cells and the effect of this process on the rate of glycolysis are being investigated.

SUMMARY

The hexokinase (HK) activity of ascites tumor cells was found to increase when a cellular suspension was incubated in vitro at 37°. The kinetics of the increase of HK activity were studied on cells incubated in air and in $O_2 + CO_2$ and $N_2 + CO_2$ mixtures. The lysis of the tumor cells which resulted in the later stages of incubation did not affect the observed increase in HK activity.

The HK formation in ascites cells in vitro was completely abolished by 2,4-dinitrophenol ($2 \cdot 10^{-4}$ M) and bromoacetate ($2 \cdot 10^{-3}$ M).

Ribonuclease, aminopterin and 4-aminopteroylaminoadipic acid were also found to inhibit HK formation in vitro.

HK was found to pass into the ascitic fluid during the in vitro incubation of ascites cell suspensions.

Throughout the entire period of tumor development, there was a steady increase in HK activity in the serum of the tumor-bearing animals; no such increase was observed in controls.

The results indicate that during the in vitro short-term incubation of ascitic cell suspensions there occurred intracellular formation of the enzyme hexokinase, which subsequently passed into the ascitic fluid.

Received December 14, 1957

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THE EFFECT OF ANALOGS OF PHENYLALANINE ON GROWTH OF THE YEAST SACCHAROMYCES CEREVISIAE

V. Gabermann

Chair of Medical Chemistry, Medical Faculty of Karlov University, Plzen', Czechoslovakia

The inhibiting effect of some amino acid analogs on the growth of microorganisms has long been considered to be the result of the difficulty of including the corresponding amino acids in proteins or a hindering of the biosynthesis of proteins. Recently, papers have appeared which show that some amino acid analogs are included in bacterial proteins [1-3]; in some cases peptides were isolated from proteins and they contained the corresponding analogs [4,5]. Very interesting experiments in this connection are those which concern the initial activation of tryptophan and some of its analogs by specific tryptophan-activating enzymes from the pancreas [6]. Such activation is the first necessary condition for the inclusion of the amino acids in the protein molecule and is accomplished by the biosynthesis of aminoacyladenylate, fixed on the surface of the activating enzyme. Those analogs which are activated by the enzyme mentioned are included in the bacterial protein and hinder growth only after several cell divisions. Some analogs inhibit the activating enzyme by forming an inactive complex with it; these analogs are not included in the protein and hinder growth at once [7].

The mechanism of the effect of the analogs of amino acids can thus be of two kinds. In one case biosynthesis of proteins is actually inhibited, as was originally assumed. In the other case biosynthesis as such is not inhibited—an atypical protein is synthesized which contains along with the amino acid, its analog. Such a protein has different physicochemical properties; in most cases it is metabolically inert, and it leads finally to a slowing or prevention of growth.

Our experiments also indicate the formation of such an atypical protein.

EXPERIMENTAL PART

The yeast Saccharomyces cerevisiae was grown at 30° on a basic medium which contained 0.2% glucose, 0.2% KH_2PO_4, 0.2% Na_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2$, and 0.2% $(NH_4)_2SO_4$. To 100 ml of medium we added 1 ml of vitamin solution (100 ml of solution contained 0.4 mg nicotinic acid, 0.4 mg thiamine, 10 mg inositol, 0.4 mg calcium pantothenate, 0.4 mg pyridoxine, 0.2 mg p-aminobenzoic acid, and 0.4 mg riboflavin) and 0.2 ml of a solution of microelements (the solution contained in 100 ml, 500 μg $MnSO_4 \cdot 4H_2O$, 200 μg $ZnSO_4 \cdot 7H_2O$, 100 μg $CuSO_4 \cdot 5H_2O$, 400 μg $FeSO_4 \cdot 7H_2O$). If necessary, the pH was brought to 5.6 with hydrochloric acid. For inoculation we used 18-hour cultures, twice washed with a basic medium which did not contain a source of nitrogen.

For the experiments, the yeast was grown in media in which the ammonium sulfate was replaced by the analog at a final concentration of 0.005 M as the sole source of nitrogen. In these cases the media were not sterilized, in order to exclude the possibility of artifacts due to oxidation of the analogs. Incubation was always carried out in a thermostat in parallel for 15-20 experiments.

We studied the analogs DL-2,4-dichlorophenylalanine (DCP), DL-p-methoxyphenylalanine (MOP), DL-chlorophenylalanine (CP), and DL-α-methylphenylalanine (MP), which were kindly supplied by A. M. Iurkevich (1st Moscow Medical Institute); the other preparations and reagents were commercial (for analysis) and were used within without further purification.

The growth of the yeast was recorded by turbidity measurements on a Coleman spectrophotometer at 450 mμ, the protein was determined in a micromodification of the Nessler reaction [8]. Free electrophoresis was carried out in Kern apparatus LK-30 in 0.01 M Na_2HPO_4. Samples for electrophoresis were prepared as follows. The yeast was centrifuged, washed with cold acetone and ether, and then extracted with 0.01 M Na_2HPO_4. The extraction was carried out for 48 hours with stirring in a refrigerator. The extract was centrifuged, dialyzed for 24 hours against the same buffer solution, and then after determination of its protein content, it was diluted so that in all the samples there was the same content of nitrogen compounds. Electrophoresis was carried out at 90 v and 2.3 ma for 60 min.

Determination of L-3,5-diiodotyrosine (DIT) in the protein was carried out as follows. The acetone preparation of the yeast was extracted three times within 12 hours with cold 5% solution of trichloroacetic acid and then was washed with alcohol. We hydrolyzed this preparation 18 hours at 110° with 6 N HCl in a sealed capillary; the hydrolyzate was evaporated over potassium hydroxide, the residue was dissolved in water, and studied chromatographically. We used Whatman paper No. 1 and the solvent n-butanol-ethanol-water (4 : 1 : 1); the results were developed with a 0.1% solution of minhydrin in acetone.

RESULTS

We were able to establish several groups (Fig. 1) depending on the effect of the analog on yeast growth. DCP and DIT strongly hindered growth; it became linear and finally almost stopped. MP stopped growth entirely. CP somewhat increased the latent period; the rest of the growth curve resembled that for tyrosine.

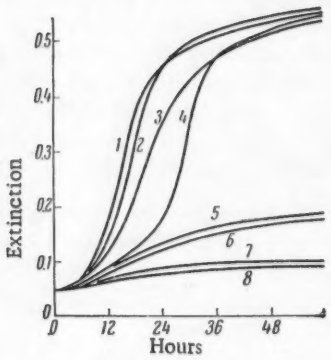

Fig. 1. Growth of yeast on media which contain different amino acids and their analogs as the sole nitrogen source. Growth measured every 6-8 hours. 1) MOP; 2) tyrosine; 3) phenylalanine; 4) CP; 5) DIT; 6) DCP; 7) control without nitrogen source; 8) MP.

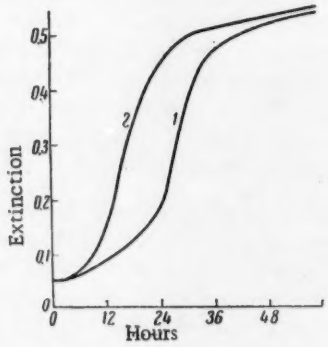

Fig. 2. Adaptation of yeast to CP. 1) Unadapted yeast; 2) adapted yeast.

MOP did not inhibit growth and the growth curve was the same as the growth in a tyrosine medium. The growth curve with L-tyrosine rose more steeply than when DL-phenylalanine was used. We do not believe that this difference resulted because we used the natural L-amino acid and in the other the synthetic racemate, DL-phenylalanine; in the control experiments with L- and DL-alanine we found no difference in the growth curves.

Biochemical degradation of phenylalanine is brought about through tyrosine, and therefore the use of the latter makes the utilization of nitrogen by the yeast easier.

Lengthening the latent period after inoculation into a medium which contains CP as the sole nitrogen source apparently produces biosynthesis of adapted enzymes. These yeasts grown on media with CP give a normal latent period when inoculated into a fresh medium of the same composition.

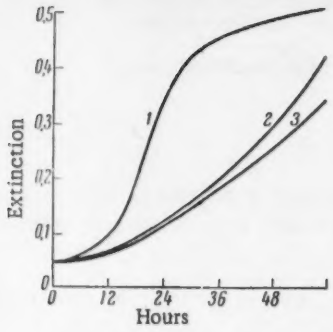

Fig. 3. Growth of yeast raised on media with analogs, after inoculation onto a basic medium with ammonium sulfate. 1) MP culture; 2) DIT culture; 3) DCP culture.

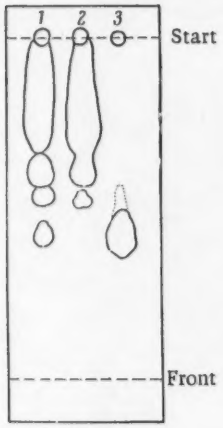

Fig. 5. Diagram of chromatogram of protein hydrolyzate from yeast. 1) Hydrolyzate of 3,5--diiodotyrosine culture; 2) hydrolyzate of phenylalanine culture; 3) control, 3,5-diiodotyrosine.

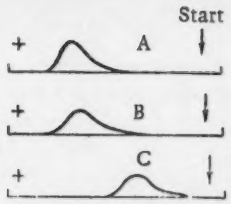

Fig. 4. Free electrophoresis of protein extracted with 0.01 M Na_2HPO_4: A) Phenylalanine culture; B) p-chlorophenylalanine culture; C) 3,5-diiodotyrosine culture.

We can assume that the analogs DCP and DIT are included in the protein molecule with the formation of poor and atypical proteins. After inoculating these cultures on fresh medium which contained ammonium sulfate we found that the growth curves rose very slowly (Fig. 3). When we added MP, growth stopped at once, probably because of the impossibility of using this substrate or the inhibition of some enzyme; when we inoculated it onto a fresh medium with another nitrogen source, we observed only some increase in the latent period (Fig. 3).

The assumption of formation of atypical proteins after growth on media with DCP is supported by the fact that in this case a colony of unusual form appears: large granules with a diameter of 1.5 mm. Microscopically we do not find differences in the structure of individual cells.

We are more interested in the detailed effects of DIT. Inhibition of growth is not due to the action of the same concentration of iodide. Oxidation of this analog by long sterilization at 3 atm. causes loss of inhibiting properties.

When we compare the amount of protein of yeast grown in the presence of different analogs in the previous experiments, we find some differences. First of all, under our experimental conditions we extracted less protein with 0.01 M Na_2HPO_4 from 100 mg of acetone preparation of yeast raised in the presence of DIT than in the other cases.

We obtained the following data

Analog	Extracted nitrogen in mg per 100 g of acetone preparation (after dialysis)
DL-phenylalanine	0.78
L-tyrosine	0.81
CP	0.77
DIT	0.52

In free electrophoresis we found less mobility of the protein from yeast grown in the presence of DIT. This protein migrated toward the anode more slowly than the other proteins which we studied (Fig. 4).

In the chromatogram we found, in the hydrolyzate of the protein grown in the presence of DIT, a considerable amount of this amino acid (Fig. 5).

SUMMARY

The yeast Saccharomyces cerevisiae includes DIT and probably DCP in its proteins. The resulting proteins which contain these analogs have different physicochemical properties and cannot take part in normal metabolism, which in the end results in slowing and cessation of growth.

Probably yeasts cannot use the nitrogen of MP and cessation of growth is due to nitrogen starvation. This analog can play a definite part in the reaction with some enzyme systems.

MOP is apparently not included in proteins, but is very easily decomposed; from the nitrogen which is liberated the yeast synthesizes needed amino acids.

The relation of yeast to CP is very complex; apparently the yeast protein does not include this analog but it can be split by yeast only after a definite adaptation.

We express our deep thanks to Candidate in Chemical Science A. M. Iurkevich, our co-worker in the biochemical laboratory of the 1st Moscow Medical Institute, for kindly synthesizing the analogs for this work.

Received January 20, 1958

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ENZYMATIC METHODS IN THE STUDY OF THE STRUCTURAL PECULIARITIES OF VARIOUS DEXTRANS

E. L. Rozenfel'd

Department of Physiological Chemistry, The USSR Academy of Science, Moscow

The deciphering of the fine structure of glycogen-type polysaccharides became possible only after Kurt Meyer et al. [1], and later Stepanenko and Afanas'eva [2-4], Swanson [5], Cori and Larner [6] and others began to apply enzymatic methods, along with the conventional chemical ones, in the study of the glycogen molecule. The chemical methods (methylation and periodate oxidation) permitted detection of the various bonds in the polysaccharide molecule, the "chain units" and the "degree of branching". The length of the terminal branches of these molecules and the structure of their "cores" could only be elucidated by combination of the chemical methods with the specific enzymatic ones, namely, by the sequential action of the enzymes catalyzing the hydrolysis of α-1,4-glucosidic links in the terminal branches (phosphorylase, β-amylase), and of those which hydrolyze the α-1,6-glucosidic links, that is, the branching links (various glucosidases).

In the dextran molecules, as distinct from those of glycogen, the glucose residues are bound in α-1,6--linkages, the branching links being either 1,3- or 1,4.

However, up to date the structure of the dextran-type polysaccharides has received considerably less attention than that of glycogens. The application of methylation, periodate oxidation, partial hydrolysis and infrared spectroscopy produced some data on the presence of various bonds, their content in the molecules of various dextrans, and the degree of branching. There is, however, no precise information on the structure of dextrans. The various models of dextran structures proposed by a number of authors (tree-shaped, laminated, comb-shaped etc.) are quite arbitrary and are not based on experimental evidence [7].

The various bacterial dextranases, known as endodextranases, whose action on dextrans results in a number of large molecular fragments, as well as isomaltose, isomaltotriose and some higher saccharides, are not suitable for the study of dextran structure. Sery and Hehre [8] have demonstrated the presence in the animal intestine of two enzymes of bacterial origin, capable of hydrolyzing dextran; their mechanism of action was dissimilar. The authors did not obtain a separation of the two enzymes.

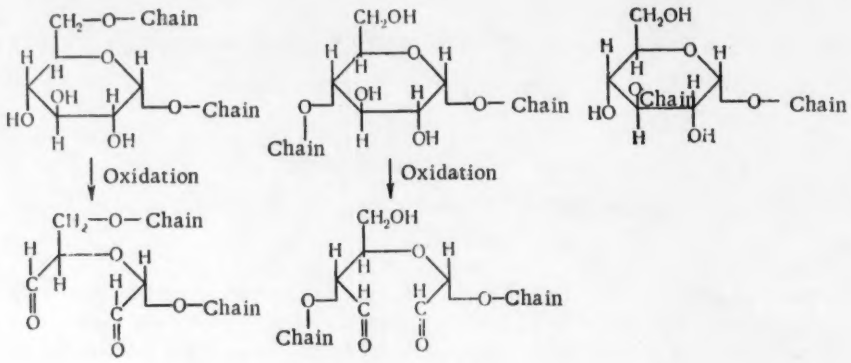
The enzyme α-1,6-dextran glucosidase, capable of cleaving the terminal glucose moieties of the dextran up to the branching point, with the formation of free glucose and residual dextran, has recently been isolated from various animal tissues [9, 10]. This enzyme seemed to present a suitable tool in the investigation of certain peculiarities in the structure of various dextrans.

Some "clinical" dextrans were used in the study of their structure and their attackability by α-1,6-dextran-glucosidase. The preparations employed were, a Swedish one, Macrodex, British – Intradex, Soviet – Polyglukin and Polish -- Polyglukan.* The molecular weight of these preparations, which showed considerable polydispersion, is known to lie between 40,000 and 80,000.

Information about the chemical structure of the preparations used was obtained by the application of periodate oxidation according to the procedure of Rankin and Jeanes [11]. Formic acid, resulting from the oxidation of dextran by periodic acid, was estimated by titration with barium hydroxide. The amount of periodate

*The preparation of Polyglukan was kindly supplied by Dr. K. F. Murawski, who also communicated useful data on its structure and molecular weight.

utilized was determined according to Fleury and Lange [11]. Periodate or periodic acid oxidation allows the estimation of the number of 1,6-and other bonds in the dextran molecule. The oxidation of glucose residues linked in the 1,6-bond results in the liberation of one mole of formic acid, and the utilization of two molecules of periodate, to each mole of glucose oxidized. During the oxidation of one mole of glucose residue linked in the 1,4-bond, one mole of periodate is utilized, but no formic acid is formed. Glucose molecules linked in the 1,3-bond are not oxidized by periodate:



	1,6-bond	1,4-bond	1,3-bond
IO_4^- used in moles	2	1	0
HCOOH formed in moles	1	0	0

This allows the calculation of the proportionate content of the 1,6-and other bonds in the molecules:

$$\% \text{ of 1,6-bonds} = M \cdot 100$$
$$\% \text{ of 1,4-bonds} = (P - 2M) \cdot 100$$
$$\% \text{ of 1,3-bonds} = 100 - [(M \cdot 100) + (P - 2M) \cdot 100],$$

where P is the number of moles of periodate used, and M – the number of moles of formic acid formed, per mole of glucose residues oxidized.

The easily soluble sodium salt of periodic acid is usually used for the oxidation of polysaccharides. For successful periodate oxidation of dextrans the solution should contain about 3 moles of periodate per mole of glucose residues.

TABLE 1

Periodate and Formic Acid Content after the Oxidation of Dextran for 72 and 96 Hours
(preparation aliquots – 90 mg, moisture – 16.6%)

Determination	72 hours			96 hours		
	0.01 M $Ba(OH)_2$ in ml		formic acid in moles per mole of glucose residues	0.01 M $Ba(OH)_2$ in ml		formic acid in moles per mole of glucose residues
	control	experiment		control	experiment	
Formic acid content	0.08 0.08	5.17 5.18	0.87	0.14 0.14	5.23 5.30	0.87
	0.1 M I_2 in ml		periodate in moles per mole of glucose residues	0.1 M I_2 in ml		periodate in moles per mole of glucose residues
Periodate content	3.80 3.81	4.94 4.93	1.92	3.83 3.85	4.95 4.96	1.89

Dextran aliquots (about 80-90 mg) were dissolved in 40 ml of boiled distilled water in flasks fitted with ground-glass stoppers, and 40 ml of 0.0347 M sodium periodate solution was added to each flask; blank vessels, containing no dextran, were set up concurrently. The solutions were incubated for 72 and 96 hours at 25°, after which period the content of formic acid and of periodate in each vessel was determined. Oxidation was usually completed within 72 hours, and no further formate formation or periodate uptake could be detected in the vessels incubated up to 96 hours (Table 1).

The moisture content of all dextran preparations was determined separately.

Formic acid was estimated by titration with 0.01 N $Ba(OH)_2$ after the excess periodate was destroyed by the addition of ethylene glycol. The titration was carried out under nitrogen. Periodate was estimated by reduction with excess sodium arsenite. Unused sodium arsenite was back-titrated with 0.1 N iodine solution. The concentration of the $NaIO_4$ in the solution was calculated from the amount of sodium arsenite used: one ml of the 0.1 N sodium arsenite solution corresponded to 0.010696 g of $NaIO_4$.

Formic acid estimation. Ten ml of the solution was treated with 0.3 ml of ethylene glycol and allowed to stand in the dark for one hour. The aliquot was then titrated with 0.01 N $Ba(OH)_2$ solution, using phenolphthalein as an indicator. The flasks with the ground-glass stoppers, which were used for titration, were initially filled with nitrogen. The entire titration was also carried out in a nitrogen atmosphere.

TABLE 2

The Content of 1,6-Bonds in Various Dextran Preparations

Preparation	Amount, in moles per mole of glucose residue		Number of bonds in the dextran		Molecular weight
	formic acid formed	periodate uptake	1,6-bonds	other bonds	
Macrodex	0.87	1.87	87	13	~60,000
Intradex	0.92	1.90	92	6	—
Polyglukin	0.97	1.98	97	4	~60,000**
Polyglukan	0.88	1.87	88	11	~50,000*

*Data by K. F. Murawski.

**Data by G. Ia. Rozenberg.

Periodate estimation. To 5 ml of the solution was added 10 ml of saturated $NaHCO_3$ solution, followed immediately by 5 ml of 0.1 N sodium arsenite, and the mixture was allowed to stand in the dark for 15 min; one ml of a 20% KI solution was then added and the mixture titrated with 0.1 N iodine solution in the presence of starch. The iodine solution was standardized with biiodate ($KIO_3 \cdot HIO_3$), and the $Ba(OH)_2$ solution — with succinic acid.

The results of the quantitative determination of 1,6-bonds in various dextran preparations are presented in Table 2.

It will be seen that Polyglukin contained the highest amount of 1,6-bonds — 97% —, while Intradex contained somewhat less, and Polyglukan and Macrodex contained a still lower, and somewhat similar, proportion of 1,6-bonds — 87-88%.

All the preparations of the clinical dextrans in hand were then digested with 1,6-dextranglucosidase.

The degree of splitting of the various preparations by the enzyme is shown in Table 3.

It will be seen from the Table that Polyglukan was split by the dextranglucosidase to a considerably lesser extent than Macrodex. At the same time, judging from the results of periodate oxidation, the numbers of branching points in the two dextrans were approximately equal. It follows that the structures of the two dextrans differ in that the terminal chains in the Polyglukan molecule are shorter than those of Macrodex. On the other hand, the British and Swedish preparations, although split to the same extent by the dextranglucosidase, contained different proportions of 1,6-bonds. This may be explained by the combination of two factors — the

presence of different numbers of side chains of different lengths (a larger number of shorter chains in Intradex and a smaller number of longer chains in Macrodex).

TABLE 3

The Splitting of Dextrans by α-1,6-Dextranglucosidase.

Incubation mixture: 10 ml of the dextran solution + 0.5 ml of acetate buffer, pH 4.8, + 2.0 ml of the enzyme solution. incubation – 48 hours at 37°

Preparation	% of dextran hydrolyzed
Macrodex	30 – 35
Intradex	30 – 35
Polyglukin	21 – 24
Polyglukan	14 – 18

The length of the terminal chains of a polysaccharide is known to be of particular importance in its protein complexing abilities [12]. The presence of shortened terminal branches on β-amylase-treated glycogen, or of short terminal branches on some dextrans [13, 14], interfere with their complex formation with proteins. Certain structural peculiarities of dextrans also affect considerably their immunogenic properties [14].

The application of 1,6-dextranglucosidase for the study of the structural peculiarities of various dextrans seems to present promising possibilities.

SUMMARY

The degree to which various dextrans were split by α-1,6-dextranglucosidase was found to depend on the number of branching points in the dextran molecule and on the length of the terminal side chains.

By the use of the periodate oxidation method and by the determination of the dextrans lability to α-1,6-dextranglucosidase, it was possible to ascertain the number of branching points in various dextrans (some possessing similar degree of polymerization) and to establish the fact that the terminal branches of Macrodex (a Swedish dextran preparation) molecules were approximately twice the length of those of Polyglukan (a Polish dextran preparation).

Received February 3, 1958

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**In Russian.

BOOK REVIEW

M. A. Dobrinskaia and N. A. Pavlovich

A TEXTBOOK OF BIOLOGICAL CHEMISTRY
(For Medical Students). Medgiz, 1957, 175 pp.

All of the available biochemistry textbooks published in this country have been designed for the use by university students and, because of their volume and contents, are not accessible to medical school students. For that reason the appearance of a biochemistry textbook for medical schools is a particularly welcome event. The authors have succeeded in presenting in the slim volume – 175 pages – the main aspects of descriptive and dynamic biochemistry in a simple and comprehensible language. However, the brevity and simplicity of any textbook should under no circumstance be allowed to affect its scientific value. In this respect certain objections may be raised against the "Textbook of Biological Chemistry" by Dobrinskaia and Pavlovich.

The nucleoprotein section (p. 78) is inexcusably brief, there being no mention about the biological role of nucleoproteins, or about filterable viruses. The additional information could have been included into the book without increasing its general volume, through space-saving cutting of some repetitions in the text, viz., the repetitive presentation of almost identical fat formulae (pp. 50 and 52).

The fairly detailed description of the isolation and purification of enzymes (p. 16) would have benefited from the addition of at least one sentence mentioning the crystallization of enzymes – one of the greatest achievements of modern enzymology.

There is no mention, in the section dealing with β-oxidation of fats, of the role of CoA in this process and of the formation of acetyl-CoA (p. 59). Some short mention of CoA should have been made in view of the great importance of this coenzyme that has emerged in the recent years.

There is no mention in the book of any transamination reactions, the importance of which in protein metabolism could hardly be overestimated. At the same time, almost two pages have been devoted to various pathways of deamination.

The tendency for keeping the volume to a minimum could also hardly provide an excuse for inaccurate statements, viz., on p. 19, on the role of carbohydrates in the body. This is alleged to consist in providing an energy source and prompting the normal oxidation of fats. The importance of carbohydrates and their derivatives as plastic, building material in the synthesis of other compounds was ignored. No mention was made about the importance of mucopolysaccharides and other polysaccharides in the protection of the body from infections, in the prevention of toxin penetration, in fertilization processes, in tissue regeneration and in the regulation of enzymatic reactions.

There are a number of other small inaccuracies in the textbook reviewed. Even a medical school graduate would benefit from the knowledge that amylase hydrolyzes not carbohydrates generally, as stated on p. 14, but only two types – starch and glycogen. It is also incorrect to refer to amylose and amylopectin as "ester-type combinations of carbohydrates with phosphoric acid" (p. 33). It is known that in wheat and other Gramineae the phosphoric acid forms no chemical bond at all with the carbohydrate component of the starch, while in the potato tuber ester bonds are apparently formed only between phosphoric acid and amylopectin.

The denaturation phenomenon has been defined on p. 65 as an "irreversible coagulation of the protein," while, according to modern views, denaturation frequently results in the opposite.

"The activities of enzymes are at their highest at temperatures between 37 and 43°" – the authors declare on p. 12 – "and complete loss of catalytic activity of the enzymes occurs between 60 and 70°." However, the temperature optimum of 37-43° is true only for the enzymes of warm-blooded animals. At the same time it is possible to name a large number of enzymes whose temperature optima lie within the 50-60° range.

It is stated on p. 24 that ". . . galactose is formed as a result of the decomposition of more complex carbohydrates . . .," although galactose may be formed from glucose by the action of galactowaldenase. The figures 17,400 and 6800* are quoted as examples of high molecular weights of proteins (p. 63). This is obviously questionable, since proteins with considerably higher molecular weights are known. Glucosides are not formed from glucose alone (p. 27), since other monosaccharides also participate.

The nearly complete identity of some of the passages in the reviewed textbook, with those in the textbook "Biological Chemistry" by Zbarskii et al. (Medgiz, 1954), seems somewhat surprising; as instances may be quoted pp. 11, 39, 80, 161 and 162 of the reviewed book, and respectively, pp. 120, 299, 361, 232 in the book of Zbarskii et al.

In view of the above, it is hoped that, should another edition of the "Textbook of Biological Chemistry" by Dobrinskaia and Pavlovich be contemplated, the book would be seriously revised and supplemented.

A. A. Anisimov

*As in original.

ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Eksptl. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratorii	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Eksptl. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariia	Veterinariia	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniia	Voprosy Pitaniia	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaia Laboratoriia	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiia	Journal of General Biology
Zhur. Vysshei Nerv. Deiatel.	Zhurnal Vysshei Nervnoi Deiatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

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